IDENTIFICATION, CHARACTERIZATION, AND MOLECULAR CLONING OF THE IMMUNODOMINANT *BABESIA BOVIS* MEROZOITE SURFACE PROTEIN Bv42

By

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KEY TO ABBREVIATIONS

PCV, packed cell volume

IFA, (indirect) immunofluorescence assay

MASP, microaerophilous stationary phase (*Babesia bovis* cultures)

IV, intravenous

iRBC, infected erythrocytes

PBS, phosphate buffered saline

NP-40, Nonidet P-40

PMSF, phenylmethyl-sulfonylfluoride

nRBC, normal (uninfected) erythrocytes

VBS, veronal buffered saline

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

kDa, kilodalton

kb, kilobase

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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Eight surface-radioiodinated merozoite proteins from a cloned, pathogenic isolate of *Babesia bovis* can be immunoprecipitated by antibody from cattle that are completely protected against clinical babesiosis. Among these eight surface proteins, the 55 and 42 kDa molecules are biosynthetically labeled with ^{3}H-glucosamine. The 42 kDa glycoprotein can also be labeled with ^{3}H-myristic acid and partitions exclusively into the detergent phase in Triton X-114 extracts, indicating that it is an integral membrane protein and suggesting that it is anchored by a glycosylphosphatidylinositol moiety. Antibody mediated protection against *B. bovis* merozoites most likely requires a high level of circulating antibody to ensure antibody-merozoite binding during the parasite's brief extraerythrocytic phase. Antibodies in diluted sera

selectively recognize the 120, 85, 55, and 42 kDa surface proteins. Only the 42 kDa integral membrane protein is reactive with serum antibodies diluted $\geq$ 1:16,000. Thus, we hypothesize these immunodominant proteins, especially the transmembrane 42 kDa glycoprotein, are important to the induction of the protective immune response and are candidates for an improved vaccine against babesiosis.

A 0.9 kb cDNA fragment encoding the 42 kDa immunodominant merozoite surface antigen Bv42 was cloned into the unique Eco RI site of the bacteriophage vector Lambda ZAP II. The 45 kDa recombinant protein expressed in Escherichia coli accounted for greater than 95% of the native 42 kDa glycoprotein, including the epitope(s) defined by monoclonal antibodies that react with the surface of live merozoites. Antibodies prepared against recombinant Bv42 immunoprecipitated the native 42 kDa glycoprotein from surface labeled and metabolically labeled B. bovis. Further, antibodies against the recombinant protein bound to and removed the 42 kDa protein recognized by surface reactive monoclonal antibody Babb 35A4 from a preparation of native, ^{35}S-methionine labeled parasite antigen. The cloning of the gene for Bv42 will now allow us to test the ability of an immune response against this specific merozoite surface antigen to protect cattle against babesiosis.

CHAPTER 1

LITERATURE REVIEW AND RESEARCH STRATEGY

Literature Review

Introduction and Significance

The arthropod-borne, hemoparasitic diseases of domestic animals are significant obstacles to improved meat, milk, and fiber production in the lesser developed tropical nations, and represent a primary barrier to the importation of more productive breeds of food animals [1]. Babesiosis is a protozoal disease that is of critical importance to cattle living in or bound for the tropical and subtropical regions of the world. The two most important species, Babesia bovis and B. bigemina, are estimated to endanger 500 million cattle between the thirty-second parallel north and fortieth parallel south of the equator, where their geographic distributions correspond to that of their vectors [2,3].

Economic loss in babesiosis is attributable to high mortality, which is in excess of fifty percent for susceptible Bos taurus cattle, and to diminished production of milk and meat in animals recovering from acute infection and abortion. Additional losses include the high cost of

quarantine and other measures to control the spread of the disease, as well as the costs associated with loss of market and inability to import high yielding Bos taurus breeds [2]. In Australia, tick-borne disease costs the government and cattle producers an estimated 42 million dollars (Australian) per year [2]. Economic losses are also significant in many Latin American countries, but it is difficult to determine the economic cost of babesiosis in much of the developing world due to lack of epidemiological data [3].

In the U.S., eradication of Boophilus ticks and babesiosis is estimated to save the country 500 million dollars per year, but the potential for re-introduction of Babesia-infected ticks from Mexico, the Caribbean, and the Southern Hemisphere via illegal livestock importation remains an ominous possibility [2]. The potential for catastrophic outbreak even in areas where tick-borne disease appears to be controlled was illustrated by the epizootics that occurred during the Zimbabwean pre-independence war in the mid-1970's. Nearly one million cattle died of hemoparasitic disorders following the disruption of short-interval dipping used to control cattle ticks [4].

In Zimbabwe and many of the endemic areas, B. bovis, B. bigemina and the rickettsia Anaplasma marginale can share

the same tick vector and have vectors with shared geographic ranges. Infections often occur in combination, resulting in synergistic pathogenicity and a disease complex collectively called "tick fever." Control of this complex, including attempts to control by vaccination, will ultimately require a strategy directed against all three organisms.

Our long-range goal is an antigenically-defined subunit vaccine against Babesia bovis. The protection afforded by recovery from primary infection indicates that a vaccine is an achievable goal. Earlier work with killed, semi-defined antigen preparations illustrates the potential for a subunit vaccine that could induce a protective immune response in susceptible cattle.

Background--Life Cycle and Disease

Transmission of bovine babesiosis is by a variety of ticks, of which Boophilus species are most notable. Susceptible ticks become infected when they acquire a blood meal from an infected cow and can transmit the disease to other cattle only after the completion of a developmental cycle that begins in the tick midgut [5]. Here the majority of parasites in the imbibed blood die while a small percentage of unique intracellular forms, the putative

gametocytes, begin a metamorphosis. Although fusion or fertilization of gametes has not yet been observed, the morphologic changes that occur suggest a sexual process [6,7]. The result is a spherical body that is transformed into an elongate ookinete. Invasion of gut epithelium is followed by asexual division and the production of sporokinetes (sporogony). These motile kinetes are released by gut epithelium and disseminated via hemolymph to enter salivary gland cells, muscle fibers, haemocytes, ovarian cells and oocytes. Further asexual division in these cells produces more kinetes which remain infective only for the tick cells. Dormancy of the parasite in tick oocytes allows for transovarial transmission to subsequent generations. When progeny ticks mature and begin to feed, sporogony again occurs in a manner similar to that following primary infection in the adult. It is the invasion of salivary gland cells by sporokinetes that initiates the form of asexual reproduction that produces sporozoites which are infective for cattle. Differentiation to the sporozoite requires a feeding or temperature stimulus and the parasite is then transmitted via saliva during tick feeding.

Following inoculation into cattle, sporozoites of Babesia directly bind and enter erythrocytes. The

sporozoite becomes an intracellular feeding stage and subsequently undergoes binary fission (merogony) to form two to four daughter cells (merozoites). Merozoites exit the infected erythrocyte causing host cell destruction and are immediately infective for other erythrocytes, thereby perpetuating the cycle of red blood cell invasion and destruction.

The replicating population of Babesia in a host is heterogenous due to the presence of subpopulations that differ in antigenicity, transmissibility and virulence. Likewise, there is evidence for antigenic differences between isolates and for selection of subpopulations by passage in both invertebrate and mammalian hosts [8,9].

Clinically, bovine babesiosis tends to be acute and life-threatening, especially in Bos taurus cattle which are more susceptible to infection than Bos indicus [10,11]. Hemolysis due to intracellular parasite multiplication and subsequent erythrocyte destruction peaks at 7 to 20 days post-infection. Accompanying signs include anemia, fever, icterus, roughened hair coat, dehydration, and depression. Pregnant animals may abort and hemoglobinuria is common.

B. bovis-infected erythrocytes sequester in visceral vessels, notably cerebral and renal capillaries. Peripheral

blood parasitemias may remain below 1 percent, while parasite rates in the cerebral capillaries typically exceed 90 percent [10]. Neurologic signs are attributed to the blockage of cerebral capillaries by parasitized erythrocytes and to anemic hypoxia. Also with B. bovis infections, significant vascular changes occur when total parasite numbers are still low and large scale hemolysis has not yet developed [11]. Parasite-produced proteolytic enzymes are released into the blood stream during the exit of merozoites from infected red cells and activate certain plasma proteins, including kallikrein and components of the complement and clotting cascades [10]. The result is activation of potent vasoactive mediators and a profound hypotensive shock.

Cattle that survive the acute phase of babesiosis typically become carriers whose blood is directly infective to non-immune animals and tick vectors [11]. Although the parasite is maintained in the cattle and tick populations via these asymptomatic carriers, persistently infected animals are also resistant to clinical disease if they are challenged - a state known as premunity or "co-infectious immunity" [10]. Prolonged sterile immunity can be seen in cattle that eliminate the organism either spontaneously or due to antibiotic treatment [12,13]. This indicates that

living parasites need not be present in the blood stream for animals to be resistant to disease [10]. The protection engendered by infection is strong evidence that an effective vaccine is an achievable goal.

Current Methods of Control

The United States' eradication of *Babesia* via its tick vector has proven to be an isolated experience not likely to be repeated on a large scale [2]. Current strategies to control babesiosis in endemic areas are a combination of vector reduction and immunoprophylaxis.

Vector Reduction Tick control by interval dipping and exclusion of vectors from areas of animal congregation remains an important mainstay in the prevention of hemoparasitic diseases [3]. In many developing tropical countries, acaricide programs consume a sizable percentage of the national veterinary services budget [14]. These programs also have important health considerations for people involved in dipping, but they can effectively decrease losses due to arthropod-borne diseases. The paradox is that reduction of the tick population in endemic areas not only retards the rate of *Babesia* transmission, it also increases the number of susceptible cattle [4]. This occurs in part because calves have a relatively high

resistance to clinical babesiosis for several months after birth. Resistance is associated with poorly defined host factors and with passive transfer of colostral antibodies from immune dams [15]. Infection during this period typically results in mild or inapparent disease and the prolonged protection afforded by subclinical infection (premunition) [15]. Effective methods of tick control decrease the frequency of calfhood infection and therefore also reduce the number of cattle protected by natural infection. Subsequent changes in tick control practices in endemic regions can upset the balance between numbers of susceptible cattle and infected ticks, and result in disastrous epizootics [4].

Immunoprophylaxis Vaccination is the logical method to protect individual cattle and prevent outbreaks, especially in highly susceptible animals that originate in non-endemic areas or areas where Babesia infection rates are limited by effective vector reduction programs. An effective immunization program would increase the number of resistant animals in an endemic area, thereby decreasing the required dipping frequency and its associated economic burden while restoring the enzootic stability needed to withstand possible breakdowns in the control system [15].

Currently practiced methods of immunoprophylaxis use premunization whereby resistance to the disease is associated with the presence (at least transiently) of the parasite in the bloodstream. Premunition is achieved by (I) deliberate infection with an attenuated (non-pathogenic) strain, or (II) drug cure of an infection after a significant parasitemia has developed.

In Australia, the regional government in Queensland annually distributes nearly one million doses of an attenuated, live B. bovis "vaccine" [16]. The vaccine is produced by multiple (20-30) syringe passages through splenectomized calves and is administered as a subcutaneous dose of infected erythrocytes [17,18]. The ensuing immune response in vaccinates does not prevent multiplication of challenge organisms (10^8) in erythrocytes or some early deviations in packed cell volume or rectal temperature after experimental challenge. However, these changes are reversed by about the sixth day post-infection and most animals recover without developing overt clinical signs [19]. Protection is similar to that conferred by recovery from infection with a virulent strain transmitted by infected ticks or direct inoculation of infected blood. Like natural infection, protection persists for at least four years and is effective against virulent heterologous parasites [19].

The Australia vaccine also has a number of serious disadvantages [20]. These include the following: 1) infected blood has a shelf life of only six days; 2) variable attenuation results in clinical disease in a minority of recipient cattle; 3) the vaccine is a blood product raised in calves and can serve to transmit a number of infectious agents including other hemoparasites and Bovine Leukosis Virus; 4) the large mass of host components in the inoculum can sensitize the vaccinates to bovine erythrocytic proteins and predispose to neonatal isoerythrolysis in progeny calves; 5) passage through non-splenectomized cattle (such as the vaccinates) can result in a reversion to virulence - thereby maintaining virulent parasites in the population for transmission to susceptible individuals; 6) production and distribution requires a large centralized facility and a cold-chain; and 7) the product is not suitable for Babesia-free regions, where its use could result in introduction of the disease.

More recently, investigators have reported that cattle were protected against babesiosis after infection with an attenuated strain of B. bovis produced in vitro [21,22]. In previously unexposed cattle, the tissue culture-adapted strains produced mild clinical signs and moderate changes in erythrocyte packed cell volume. Production of a vaccine

strain of Babesia in culture would improve quality control and decrease the need for splenectomized calves but would not overcome many of the limitations inherent to premunization using infected blood. Furthermore, cultivation of hemoparasites on a large scale would be difficult.

In Israel and elsewhere, the shelf life of an attenuated inoculum has been extended by storage in liquid nitrogen but the method is unwieldy [23]. Other problems with a blood-derived, living product remain, and the low survival rate of B. bovis after freeze/thawing necessitates that large number of parasites be present in each dose - thereby increasing both the mass of bovine protein inoculated and the cost per vaccinate.

The second method of premunization involves infection of cattle with attenuated or virulent strains of Babesia followed by drug cure, often using the chemoprophylactic imidocarb [24-26]. This technique is labor intensive and requires skilled professionals to treat cattle only after a protective immune response has had time to form but before acute, life-threatening disease has occurred. A sterilizing treatment administered early in the course of the infection produces a less protective immune response than if the treatment is given after a high parasitemia [12,24]. In

addition, chemotherapeutic drugs against Babesia are both expensive and toxic.

Clearly, current methods to immunize cattle against babesiosis are seriously flawed, but the solid protection produced by these techniques is encouraging.

Experimental Nonviable and Semidefined Immunogens

Recent work on bovine babesiosis has focused on using killed or semi-defined antigens to induce protective immunity. These antigens can be classified as 1) killed whole organism vaccines derived from infected blood, 2) antigens purified from parasitized erythrocytes by affinity chromatography, and 3) soluble antigens from continuous MASP cultures of B. bovis. Immunization with such preparations does not prevent infection but can significantly reduce morbidity and mortality. None of these products is likely to become a satisfactory vaccine, but the work clearly demonstrates the potential for a subunit approach.

Whole organism vaccines

A freeze-dried suspension of killed Babesia bovis has been shown to partially protect cattle from intravenous challenge with 10^6 or 10^7 infected erythrocytes [27]. The

suspension was produced by lysis of infected cells in distilled water and recovery of parasites by centrifugation. Protection was manifested by fewer clinical signs and decreased erythrocyte destruction. Subsequently, a crude babesial antigen called infected erythrocyte antigen (IEA) was prepared by disruption of concentrated, parasitized erythrocytes in a French pressure cell [28]. Subcutaneous inoculation of this product in Freund's complete adjuvant was shown to protect Bos taurus cattle (n=4) against a heterologous strain as effectively as recovery from a tick-transmitted infection 12 months previously. Protection was less effective when the challenge dose was increased from 10^6 to 10^8 infected erythrocytes. These studies indicated that at least partial protection could be conferred with killed antigen and suggested that antigenic variants were not a formidable barrier to immunization. However, the experimental preparations tested were also contaminated with host erythrocyte antigens leading to the development of isoantibodies [28].

Considerable effort has been directed toward fractionation of crude babesial antigen and demonstration of a protective immune response derived from a semidefined or purified component [29-36]. At least two subfractions of infected erythrocytes have been shown to induce a protective

immune response that is as effective as that produced by crude antigenic material [30]. The persistent problem of eliminating host protein from vaccine preparations has been complicated by the discovery that some babesial antigens complex with host protein.

Affinity purified immunogens

Three B. bovis antigens with molecular weights of 1300, 180, and 44 kDa were purified from infected blood using monoclonal antibody affinity chromatography [37]. These antigens could be specifically localized to the parasite or infected erythrocyte by immunofluorescent staining. When splenectomized calves were immunized twice with the respective antigens in Freund's adjuvant, only the 44 kDa antigen conferred protective immunity against a homologous intravenous challenge of 10^3 organisms. Subsequently the same 44 kDa-reactive monoclonal antibody was used to isolate a 29 kDa antigen that had been further purified by gradient gel electrophoresis [38]. Two subcutaneous injections of this antigen in FCA induced a protective response in nine nonsplenectomized adult cattle that were challenged with 10^4 infected erythrocytes of the homologous, virulent strain. Whereas none of the vaccinated animals showed clinical

signs, three of the five unvaccinated controls were severely affected. No animals in either group died but the vaccinated cattle had significantly less change in PCV, body temperature, and parasitemia.

Based on the relative protection of splenectomized calves, the authors judged the affinity-purified 29 kDa antigen to be as protective as crude antigen. The results in intact adult cattle were much harder to interpret considering variations in challenge dose, quantity of immunogen and strains of B. bovis used for antigen extraction and challenge. The monoclonal antibody used to isolate the 29 kDa antigen is a labile IgM that cannot be separated from contaminating ascites proteins without severely affecting its biological properties. The resulting presence of haptoglobin in the immunosorbent allows for binding of bovine hemoglobin and subsequent elution with the babesial antigen [38]. Native 29 kDa antigen is also apparently complexed to large host molecules that may act as carriers and even alter immunogenicity. Production of sufficient purified antigen clearly remains a significant problem in testing experimental vaccines. Nevertheless, this work demonstrates the potential of a defined antigen approach to develop effective immunoprophylaxis in bovine babesiosis.

MASP culture derived antigens

Candidate protective antigens of Babesia bovis have also been studied as a by-product of recent advances in in vitro cultivation by the microaerophilous, stationary phase (MASP) technique [39,40]. Immunoelectrophoresis of culture supernatants demonstrated at least three soluble antigens with molecular weights in the range of 37-40 kDa [41,42]. These may be identical to three antigens that were previously identified in the lytic extracts of infected erythrocytes [41,29]. When monospecific rabbit antibodies against the purified culture-derived antigens were used in an immunofluorescence assay (IFA), two of the antigens (1 and 2) could be localized to the erythrocyte membrane or stroma while the third was directly associated with the parasite [43]. It is this latter antigen, designated "Antigen 3", which is postulated to represent merozoite surface coat material that is shed into the culture media when the merozoite invades an erythrocyte. Bovine antisera to B. bovis culture supernatant reacts with the merozoite surface and causes morphologic alterations in the surface coat, aggregation, and lysis of merozoites [40]. By analogy with the merozoites of Plasmodium and species of Babesia that infect rodents, this antibody should prevent parasite invasion of erythrocytes and mediate their immune

destruction by the mononuclear phagocyte system. The other two antigens are apparently precipitated as fibrinogen-associated complexes that *in vivo* may affect coagulation, fibrinolysis, and immune recognition of parasite proteins.

Immunization with soluble antigens in supernatants of MASP cultures has produced conflicting results. Partial protection was induced in four 18-month-old *Bos taurus* cattle that received two subcutaneous doses (in saponin adjuvant) and were challenged three months later with 1000 *B. bovis*-infected *Boophilus* ticks [40]. Vaccinated cattle were clinically less severely affected than identically challenged controls.

A subsequent study showed protection of 18 yearling heifers for four to six months after a similar vaccine regimen and homologous challenge by intramuscular injection of 10^8 infected erythrocytes [44]. Immunization induced marked IFA serum titers that peaked 1 week after the second vaccination and were allowed to decline to approximately undetectable levels prior to challenge. Following challenge, vaccinated animals developed significantly higher IFA titers than non-vaccinated groups, and more than 50 percent of the controls died of acute babesiosis.

In both of these trials, *Babesia bovis* parasitemias were observed in all challenged cattle, but in the tick

challenge experiment lower parasitemias were generally associated with survival [40]. Although the initial replication of challenge parasites caused a reduction in PCV in all cattle, the destruction of host erythrocytes was significantly less in the vaccinated animals that were challenged by intramuscular injection [44].

The conflicting data on culture-derived immunization has been published from Australia [45,46]. This group used an identical vaccine protocol in yearling and 2-year-old Bos taurus cattle that were challenged at 20, 70, or 179 days by intravenous inoculation of 10^8 organisms of a heterologous virulent strain. Whereas a single dose of a live attenuated vaccine strain of B. bovis provided strong protection, soluble antigens derived from MASP cultures of the same organism induced only partial protection and 6 of 12 animals required treatment to prevent death. All members of the unvaccinated or saponin-recipient control groups needed to be treated. The persistent problem of contaminating host proteins was illustrated by the detection of bovine blood group antibodies in 4 of the 5 cattle that received two doses of culture-derived antigen [45]. Even soluble B. bovis antigens harvested from heterologous rabbit MASP culture systems are initially contaminated with 8 or 9 bovine proteins and the problem is more severe for antigens

produced in bovine systems [43].

The ability of culture-derived exoantigens to protect against heterologous challenge has since been examined using Latin American isolates [47,48]. Exoantigen from a Venezuelan strain induced a significant degree of cross-protection in cattle challenged with five different geographic isolates, while exoantigen from a Mexico isolate produced a low degree of cross-immunity. Vaccination did not prevent replication of parasites but did decrease the change in packed cell volume and increase the 6 week post-challenge weight gain.

Soluble antigen from microaerophilous, stationary phase cultures of B. bovis is unlikely to find use as an acceptable, effective immunoprophylactic for bovine babesiosis. The antigen is difficult and expensive to manufacture in significant amounts and must ultimately be freed of bovine contaminants. Nevertheless this work again demonstrates the potential for nonviable parasite antigen as a protective immunogen.

Recombinant Antigens of Babesia bovis

Little progress has been made in cloning genes from Babesia species, and there is only one report describing the immune response to a recombinant B. bovis antigen [49]. A

portion of the gene encoding a 44 kDa B. bovis merozoite surface antigen was cloned into bacteriophage Lambda gt11. This clone was selected from a genomic expression library screened using a panel of monoclonal antibodies against merozoite surface antigens. Cattle immunized with the fusion protein purified from E. coli produced antibodies that reacted with the surface of live merozoites and immunoprecipitated a native protein of 44 kDa. Unfortunately, the level of antibody response among vaccinates was highly variable. The investigators are now looking for another adjuvant or method of antigen presentation that will produce a more consistent antibody titer.

In another report, a positive clone was detected in a Lambda gt11-B. bovis cDNA library screened with sera from cattle premunized with the Australian vaccine strain of B. bovis [50]. The cDNA insert was 235 bp long and contained coding sequences for only 44 amino acids. Antibodies affinity purified from bovine sera by antibody select [97] identified a high molecular weight antigen ($\geq$220 kDa) in immunoblots of infected erythrocytes. The same antibodies were used in an indirect immunofluorescent assay to locate the native molecule within the parasite in infected cells. In this study, the recombinant protein was not tested as an

immunogen.

Although several additional genes of B. bovis have been cloned, the functions of these genes and the nature of their gene products is not known [51-53]. The genes have been used, however, to establish that isolates of B. bovis are composed of heterogeneous subpopulations and to identify a highly polymorphic gene family. The authors suggested, but provided no evidence, that sequence diversity at the polymorphic locus, designated BabR, played a role in antigenic variation of the parasite. It remains to be shown that the proteins encoded by BabR or any of these other genes are relevant to protective immunity.

Summary

There is compelling evidence that a safe, effective vaccine against bovine babesiosis is an achievable goal. Recovery from natural infection, premunition by attenuated strains, and drug cure of a significant parasitemia all provide strong, enduring protection against clinical disease. Passive transfer of immune sera and gamma-globulin has been used to show this protection is mediated in large part by antibody [54,55]. Antibodies directed against surface antigens exposed during the short extracellular phases of hemoparasite infections have been shown to prevent

invasion of erythrocytes and presumably opsonize parasites for phagocytosis [56,57]. Likewise, antibodies against new erythrocyte antigens that are encoded by hemoparasite DNA and expressed on the surface of infected cells mediate the removal of intracellular stages from the circulation [56,58].

Recently, immunization with non-viable, semi-defined parasite antigens has been shown to induce a host protective response that significantly reduces mortality, overt disease, and deleterious clinicopathologic changes. The antigen-specific nature of this kind of response is demonstrated by the effects of bovine anti-sera raised against the three major antigens of B. bovis in supernatants of MASP cultures [40]. These cattle are protected against clinical disease due to a tick-transmitted homologous challenge, and their antisera react with merozoites to cause aggregation, lysis, and morphologic changes in the surface coat. The failure to exploit killed antigen as an effective vaccine reflects the complexity of the disease and the serious problems involved in obtaining sufficient quantities of parasite antigen that is well characterized and pure. Many of these problems can be overcome by the application of current molecular biology techniques.

Research Strategy

The long term goal of my work is development of a safe, effective vaccine against Babesia bovis. As a part of that goal, the specific aims of the work detailed in this dissertation were:

(1) Identification and characterization of Babesia bovis merozoite surface antigens using antibodies from cattle protected against babesiosis.

(2) Identification and characterization of cDNA clones expressing recombinant merozoite surface antigens in Escherichia coli.

A prerequisite for an antigenically defined vaccine against B. bovis is the identification of parasite antigens which are necessary or sufficient for the induction and expression of a host-protective immune response. Toward that end, I identified and characterized candidate host-protective antigens located on the surface of the merozoite of B. bovis. These antigens were radioiodinated on live merozoites by a surface specific method (lactoperoxidase) [59-61] and immunoprecipitated using antibodies from cattle proven to be protected against babesiosis.

Kinetic models predict that merozoite surface antigens which are relevant to protective immunity must induce high levels of antibody to ensure antibody-merozoite binding during the parasite's brief extracellular phase [62]. Of eight surface proteins identified using antibodies from protected cattle, four were shown to be immunodominant blood stage antigens. A 42 kilodalton (kDa) glycoprotein, designated Bv42, elicited the highest antibody titer. I hypothesized that the immunodominant surface proteins, especially Bv42, were important to the induction of the protective immune response and set out to clone the gene(s) encoding one or more of these candidate immunogens.

Polyadenylated mRNA collected from asynchronous MASP cultures of a cloned B. bovis isolate was used to construct a cDNA expression library in E. coli [63-66]. cDNA was synthesized by the Gubler/Hoffman technique, which is highly efficient for producing full length cDNA copies of isolated mRNA [67]. cDNA was cloned into the unique Eco RI site in the Lac Z gene of bacteriophage Lambda ZAP II (Stratagene, La Jolla, CA, USA).

Lambda ZAP II bacteriophage was chosen as a cloning vector for the following reasons: 1) lambda phage is highly efficient for in vitro packaging and introduction of DNA into a bacterial host [69], (2) Lambda ZAP II has a multiple

cloning site which simplifies subcloning of the insert, especially if it contains an internal Eco RI site, (3) addition of a helper phage to Lambda ZAP II allows *in vivo* excision of a circularized phagemid which contains the insert and can be rescued for plating onto *E. coli* [70], (4) DNA inserted into the Eco RI cloning site of Lambda ZAP II is expressed as a fusion protein containing only 3.9 kDa of vector encoded polypeptide, and (5) the *E. coli* host strain carries the lac repressor gene, lac I, so that the potentially deleterious expression of recombinant genes can be conditionally down regulated.

Recombinant plaques were immobilized on nitrocellulose membranes and screened for the expression of immunodominant merozoite surface antigens using polyclonal, monospecific rabbit sera [71]. A clone encoding the immunodominant merozoite surface protein Bv42 was selected for further characterization. In order to prove definitively that the gene expressed in *E. coli* encoded Bv42, antibodies were raised against the recombinant antigen in rabbits and cattle and shown to recognize the native molecule.

A recombinant merozoite surface protein proposed for immunization should bear surface epitopes to induce a protective immune response against *B. bovis* merozoites. Importantly, the recombinant Bv42 protein from *E. coli* is

recognized by Babb 35A4, a monoclonal antibody against native Bv42 that reacts with the surface of live merozoites and thus defines a surface exposed epitope [72]. This observation indicates that the recombinant protein closely reproduces at least one surface exposed region of native Bv42.

CHAPTER 2

MOLECULAR CHARACTERIZATION OF *BABESIA BOVIS* MEROZOITE SURFACE PROTEINS BEARING EPITOPES IMMUNODOMINANT IN PROTECTED CATTLE

Summary

Eight surface-radioiodinated merozoite proteins from a cloned, pathogenic isolate of *Babesia bovis* can be immunoprecipitated by antibody from cattle that are completely protected against clinical babesiosis. Among these eight surface proteins, the 55 and 42 kDa molecules are biosynthetically labeled with ^{3}H-glucosamine. The 42 kDa glycoprotein can also be labeled with ^{3}H-myristic acid and partitions exclusively into the detergent phase in Triton X-114 extracts, indicating that it is an integral membrane protein and suggesting that it is anchored by a glycosylphosphatidylinositol moiety. Antibody mediated protection against *B. bovis* merozoites most likely requires a high level of circulating antibody to ensure antibody-merozoite binding during the parasite's brief extraerythrocytic phase. Antibodies in diluted sera selectively recognize the 120, 85, 55, and 42 kDa surface proteins. Only the 42 kDa integral membrane protein is reactive with serum antibodies diluted $\geq$ 1:16,000. Thus, I

hypothesize that these immunodominant proteins, especially the transmembrane 42 kDa glycoprotein, are important to the induction of the protective immune response and are candidates for an improved vaccine against babesiosis.

Introduction

Animals that survive natural field infection or that recover from infection with an attenuated strain are protected against clinical disease [19,74]. These observations indicate that the host can mount a protective immune response after exposure to parasite antigens. Merozoite surface proteins are important in the pathogenesis of babesiosis due to their role in the parasite's recognition of, attachment to and penetration of host erythrocytes [75]. Accessible to the immune system and present on the disease causing stage, these antigens are potential targets of the protective immune response and, therefore, candidates for vaccine development. The ability of merozoite surface proteins to elicit a protective immune response has been shown with Plasmodium falciparum. The precursor protein for three major merozoite surface polypeptides can induce complete protection against a lethal challenge in immunized monkeys [76]. Importantly, post-translational modification of two of these P. falciparum

proteins by glycosylation may contribute to their antigenicity [77].

Seven B. bovis merozoite surface antigens have previously been identified using monoclonal antibodies against surface exposed epitopes [72,49]. These monoclonal antibodies reacted with live merozoites in indirect immunofluoresence assays, and immunoprecipitated radiolabeled proteins of 16, 37, 42, 44, 60, 85, and 225 kDa. In this study, additional methods are used to more comprehensively detect merozoite surface targets and to characterize the post-translational modification of these proteins. Importantly, a cloned isolate of B. bovis was examined in order to avoid confusion generated by intraspecific antigenic variation [51,78]. Antibodies from each of five cattle completely protected against clinical babesiosis identify immunodominant parasite antigens that I hypothesize are relevant to the protective immune response.

Materials and Methods

Immune animals A Mexico isolate of B. bovis was cloned in vitro by limiting dilution as described [66]. Five 4- to 5-month old Holstein steers were each infected by intravenous (IV) subinoculation of 5×10^7 infected erythrocytes (iRBC) from a splenectomized calf. The cattle were challenged

three times IV with 10^8 iRBC each, once by subinoculation and twice using culture derived parasites. At 127 days post inoculation, the five cattle and three weight-matched, previously uninfected controls were challenged IV with 10^9 iRBC from culture. The packed cell volume and parasitemia of all animals was monitored daily. Sera used in the following experiments was collected at 126 days post inoculation.

Microaerophilous stationary phase cultures \ Metabolic labeling The cloned isolate was cultured in 24-well plates or flasks by a modification of a previously described method [39]. For metabolic labeling, parasites were grown for 12 hours in complete medium or methionine-deficient medium. At 12 hours the media were replaced with media containing 200 or 400 uCi ^{35}S-methionine, ^{3}H-glucosamine, or ^{3}H-myristic acid. Cultures were harvested at 24 hours and iRBC's were washed five times in cold phosphate buffered saline (PBS) prior to extraction in lysis buffer [50 mM Tris pH 8.0, 1% (v/v) Nonidet P-40 (NP-40), 5mM EDTA, 0.1mM N-alpha-p-tosyl-L-lysyl-choromethylketone, and 1mM phenylmethyl-sulfonylfluoride (PMSF)]. No radiolabel was incorporated into protein in uninfected MASP control cultures that received radioisotope.

Merozoite Purification A high percentage of parasitized erythrocytes was produced *in vitro* by progressively reducing the packed cell volume [79]. Merozoites were purified by a previously described technique [80]. Viability was assessed by mixing merozoites with 6-carboxyfluorescein diacetate. This method, previously used for demonstrating viability of *Babesia sp.* [72,81], relies on an intact cell membrane to prevent leakage of fluorescein from the cell [82]. Viability was expressed as the percentage of total parasites that were fluorescent.

Surface radioiodination Merozoite surface proteins were radioiodinated using lactoperoxidase as previously described [83]. Normal red blood cells (nRBC) from uninfected control cultures were collected, washed three times in PBS, and radiolabeled identically. An equivalent number of nRBC ghosts were prepared by lysing washed uninfected cells from control cultures by freeze/thaw in liquid nitrogen. Ghosts were washed free of hemoglobin in PBS by multiple centrifugations at 35,000 X g, 20 min., 4°C and discarding the supernatant until it was clear. The final pellet was resuspended in PBS and radiolabeled with ^{125}I using lactoperoxidase.

Immunoprecipitation Immune sera collected one day prior to the final challenge experiment were adsorbed three times with an equal volume of packed intact nRBC's and three times with an equal volume of nRBC ghosts (prepared as described above). Radiolabeled B. bovis lysate was ultracentrifuged, sonicated, and filtered as described previously [83]. This antigen was then incubated overnight at 4°C with 15 ul of bovine serum or 15 ul of a dilution of serum in veronal buffered saline (VBS) pH 7.4, containing 1% (v/v) NP-40. One hundred and fifty microliters of 10% (v/v) formalinized Protein G-bearing Streptococcus (Omnisorb@; Calbiochem; San Diego, CA) in VBS pH 7.4, 1% (v/v) NP-40, 0.1% (w/v) gelatin were added and incubated for 2 hours at 4°C [84]. The precipitates were washed twice with VBS, 1% (v/v) NP-40; four times with VBS, 2 M NaCl, 1% (v/v) NP-40, 10 mM EDTA; and twice more with VBS, 1% (v/v) NP-40. The precipitated protein was eluted by boiling in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 2% SDS and 2.5% 2-mercaptoethanol [83].

SDS-PAGE Immunoprecipitates were electrophoresed under reducing conditions in 7.5 to 17.5% continuous gradient polyacrylamide gels [83,85].

Phase separation of Triton X-114 Washed iRBC's from ^{35}S-methionine labeled cultures were lysed in 10 mM Tris, 154 mM NaCl pH 7.4, 1% (v/v) Triton X-114, 1mM PMSF at 0-4°C and frozen at -20°C. For protein separation, the antigen extract was first ultracentrifuged, sonicated and filtered as previously described [83]. The Triton X-114 solubilized proteins (10^7 protein bound counts per minute in a volume of 2.0 ml) were subjected to temperature-dependent phase separation [86]. One million protein bound counts per minute of each phase and the starting antigen preparation were immunoprecipitated as described above.

Immunoblotting Parasite antigen for immunoblotting was prepared from MASP erythrocyte cultures with approximately 25% parasitized erythrocytes. Briefly, iRBC's and nRBC controls were washed two times in cold Puck's saline G and two times in cold PBS, resuspended in PBS, counted, and frozen at -20°C. To remove hemoglobin from lysed cells the samples were thawed and washed in cold PBS (43,000 X g, 20 min., 4°C) until the discarded supernatant was clear. The final pellet was detergent extracted with lysis buffer and processed as described [83]. Twenty five million iRBC's or an equivalent total number of nRBC's (10^8) were mixed with

3X SDS-PAGE sample buffer, boiled for 3 minutes, electrophoresed in a 7.5 to 17.5% continuous gradient polyacrylamide gel, and electrophoretically transferred to a nitrocellulose membrane [87]. The nitrocellulose was blocked for 4-6 hours in VBS (pH 7.4) containing 0.25% (v/v) Tween-20, 0.25% (w/v) gelatin (blocking buffer), and reacted overnight at room temperature with immune sera diluted in blocking buffer [84]. After washing three times in blocking buffer and two times in VBS containing 0.1% (w/v) gelatin, the nitrocellulose was incubated for 2 hours at room temperature with 2.5 uCi of ^{125}I-Protein G (Amersham Corp; Arlington Heights, IL) diluted in VBS containing 0.1% (w/v) gelatin. The nitrocellulose was washed twice with VBS, 0.1% (w/v) gelatin and four times with 1M NaCl, 10 mM EDTA, 0.25% (v/v) Tween-20, and exposed to X-ray film with an intensifying screen at -70°C.

Results

Challenge of immune animals All five cattle infected and repeatedly challenged with a cloned isolate of B. bovis were completely protected against a homologous challenge. Control cattle in the final challenge experiment experienced a 28% reduction in packed cell volume (Fig. 2-1) ($p \leq .0005$ when compared to previously infected cattle; Student's

paired t test), while only the initial infection caused a significant reduction in packed cell volume in the experimental group (39%). Despite producing extensive hemolysis, this isolate seldom produces a circulating parasitemia greater than 0.1% in non-splenectomized cattle. Immune sera used in the following experiments were collected one day prior to the final challenge.

Identification and characterization of merozoite surface proteins Merozoites purified from culture were 95-100% viable by 6-carboxyfluorescein diacetate staining. Immunoprecipitation of surface radioiodinated proteins with serum antibodies from all five protected animals identified seven dominant surface proteins with relative molecular weights of 250, 120, 98, 85, 55, 42, and 37 kilodaltons (Fig. 2-2A). The 250 kDa protein does not enter the resolving gel in a standard 14 cm. 7.5-17.5% polyacrylamide gel but is clearly resolved in a 25 cm. gel (Fig. 2-2B). An eighth protein of 25 kDa was immunoprecipitated by serum antibodies from two calves (lanes 7 and 10). Control immunoprecipitation of radioiodinated intact nRBC and nRBC ghosts revealed no specific bands on SDS-PAGE (data not shown).

To confirm that these proteins were parasite derived, the immune sera were used to immunoprecipitate ^{35}S-methionine metabolically labeled parasite proteins. The immunoprecipitated ^{35}S antigen profile was identical in all five protected animals. When run side by side with ^{35}S-methionine labeled precipitated proteins, the radioiodinated 120, 98, 85, 55 and 42 kDa surface proteins comigrate with metabolically labeled antigens (Fig. 2-3). The 25 and 37 kDa proteins were inconsistently precipitated from ^{35}S-methionine labeled antigen (Fig. 2-6).

Surface protein glycosylation and myristylation were examined by immunoprecipitation of blood stage antigen after <u>in vitro</u> incorporation of ^{3}H-glucosamine and ^{3}H-myristic acid. Comigration of ^{125}I and ^{3}H-labeled antigen in a polyacrylamide gel shows that the 55 and 42 kDa surface labeled proteins are glycosylated and the 42 kDa glycoprotein is myristylated (Fig. 2-4). Neither radiolabel was incorporated into host protein in nRBC control cultures. The 34 kDa glycoprotein seen in lane 4 of Figure 4 cannot be consistently surface labeled and immunoprecipitated.

Temperature dependent phase separation of Triton X-114 was used to determine whether the surface exposed antigens were transmembrane proteins. Parasite proteins were metabolically labeled in culture with ^{35}S-methionine and

solubilized in 1% Triton X-114 at 0-4°C. The antigen preparation was warmed above the detergent's cloud point (20°C) and separated into aqueous and detergent phases by centrifugation. Immunoprecipitation from each phase and the starting solution shows that the 42 kDa antigen partitions exclusively into the detergent phase (Fig. 2-5). The other surface proteins partition into the aqueous phase.

Immunogenicity To determine the relative immunogenicity of these surface proteins in protected animals, antibodies in sequentially diluted sera were used to immunoprecipitate metabolically labeled antigen. The reactivity of these antibodies against the majority of ^{35}S-labeled proteins is lost at a 1:160 serum dilution (Fig. 2-6). However the 120, 55, and 42 kDa surface proteins remain reactive at serum dilutions of 1:160 to 1:640. Because interpretation of immunoprecipitation is dependent on the specific radioactivity of labeled proteins, antibodies in diluted sera were also examined for their reactivity with parasite antigens by immunoblotting (Fig. 2-7). Compared to undiluted serum, antibodies in immune serum diluted 1:500 recognize a limited number of blood stage proteins, including proteins that migrate at the same molecular weight as the 120, 85, 55 and 42 kDa surface antigens. A protein

that comigrates with the 42 kDa integral membrane glycoprotein is consistently recognized at serum dilutions ≥1:16,000.

Discussion

The immune response to Babesia bovis infection protects surviving cattle against babesiosis. Previous work has also shown that nonviable parasite antigens will induce partial protection in cattle, and supports a subunit approach to vaccine development [27,34,40,37]. My strategy for an improved vaccine is to identify and characterize surface antigens of the merozoite, the blood stage that is infective for the erythrocyte and accessible to the host immune system. Native and recombinant parasite antigens can then be purified, or expressed by recombinant vaccine vectors, and evaluated as immunogens.

Seven B. bovis merozoite surface proteins have been identified on an uncloned Mexico isolate using monoclonal antibodies [72,49]. By indirect immunofluorescence, these antibodies reacted with the surface of live merozoites and, therefore, defined proteins containing regions that are accessible to antibody binding. Four proteins of 37, 42, 44, and 85 kDa were distributed over the entire surface of the merozoite and could be surface radioiodinated.

In the present study, antibodies in sera from cattle that are proven to be protected against virulent challenge also react with surface iodinated proteins of 37, 42 and 85 kDa. The 42 and 85 kDa surface proteins can be immunoprecipitated using the previously descibed monoclonal antibodies (data not shown). In addition, antibodies in these sera identify five merozoite surface proteins of 250, 120, 98, 55, and 25 kDa not previously detected with monoclonal antibodies. The parasite specificity of all eight surface antigens is confirmed by the failure of serum antibodies from protected cattle to react with iodinated normal erythrocyte proteins and by the biosynthetic labeling of these proteins in cultures in which only parasite mRNA is translated [83,81]. The use of a cloned isolate in this work is significant in that all eight surface exposed proteins are expressed on the merozoite and do not represent variants in subpopulations. Uncloned B. bovis isolates, including the current Australia vaccine strain, are composed of subpopulations that differ in antigenicity, virulence and abundance within an isolate [51,78].

To induce immunity, a recombinant parasite antigen may need to be presented by a eukaryotic expression vector that can post-translationally modify the protein and preserve native epitopes including carbohydrate moieties [73].

Carbohydrate side chains are important for the antibody reactivity of Plasmodium falciparum antigens and are likely to be significant in Babesia spp. [77]. By immunoprecipitation of ^{3}H-glucosamine labeled parasite antigen, the 55 and 42 kDa B. bovis molecules are shown to be glycosylated. The contribution of carbohydrate epitopes to the immunogenicity of these proteins needs further study.

The 42 kDa merozoite surface glycoprotein was identified as a transmembrane protein using phase separation in Triton X-114. In this technique, integral membrane proteins interact with the nonionic detergent via their hydrophobic domain and when warmed above 20°C can be separated from hydrophilic proteins (which are excluded from detergent micelles) by centrifugation [86]. The ^{35}S-methionine labeled 42 kDa molecule was immunoprecipitated almost exclusively from the detergent phase. Additional surface exposed proteins that separate into the aqueous phase may be peripheral membrane proteins or may be partitioning anomalously under these conditions [88,89]. Recognized causes of anomalous partitioning of integral membrane proteins into the aqueous phase of Triton X-114 include (1) large hydrophilic moieties such as carbohydrates, (2) the formation of oligomers which protect hydrophobic domains, and (3) noncovalent association with

peripheral (hydrophilic) proteins such as those from the the cytoskeleton (90). The strong hydrophobicity of the 42 kDa glycoprotein may indicate that appropriate presentation, possibly in the context of a membrane, will be required for it to induce an effective immune response against the merozoite.

Most integral membrane proteins are anchored in the membrane by a hydrophobic stretch of amino acids. However, a significant number of eukaryotic integral membrane proteins are held in the membrane by a phosphatidylinositol lipid moiety [91]. These proteins, which include the major surface antigens of the parasitic protozoa Trypanosoma brucei, Leishmania major and Plasmodium falciparum, are covalently modified at their C-terminus with a glucosamine-containing carbohydrate chain and the fatty acid myristate [92-94]. By analogy with these protozoal surface proteins, the incorporation of both ^{3}H-glucosamine and ^{3}H-myristic acid into the 42 kDa protein suggests that this molecule is embedded in the merozoite membrane by a similar glycosyl-phosphatidylinositol anchor [95]. The previously described T. brucei and L. major anchors are sensitive to specific degradation with phospholipases, releasing the protein from the cell surface and exposing a cross-reactive epitope. Similar studies are needed to characterize the role of

myristic acid and glycans in anchoring the B. bovis 42 kDa protein.

In general, antibodies that inhibit P. falciparum merozoite invasion of erythrocytes in vitro must be present in high concentrations [62]. High antibody levels ensure that enough surface specific antibody will be available to effectively react with parasites during their short extracellular phase. Cattle that are immune to infection with a cloned isolate of Babesia bovis produce high titered antibody that is preferentially directed against four merozoite surface proteins with relative molecular weights of 42, 55, 85, and 120 kDa. The 42 kDa integral membrane glycoprotein that elicits the highest antibody titer has previously been shown to be species specific and to contain epitopes conserved in at least four geographic isolates [96 and unpublished data]. The preferential response of protected animals to these four proteins, particularly the 42 kDa glycoprotein, leads me to hypothesize that these parasite proteins are important in the induction of protective immunity. Native and recombinant protein are being isolated to test this hypothesis.

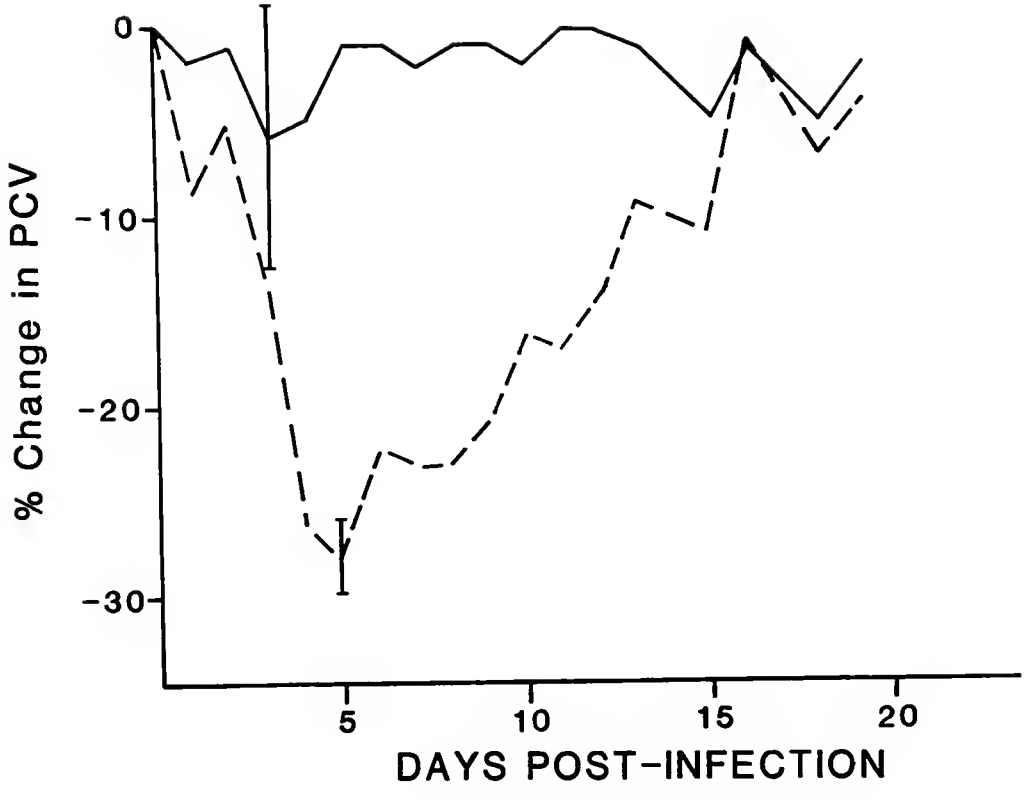


FIGURE 2-1 **Animals previously infected with the cloned Mexico isolate of B. bovis are immune to virulent challenge.** Percentage change in PCV was measured daily in previously infected (—), n=5, and uninfected (---), n=3, animals following inoculation with 10^9 infected erythrocytes of the cloned isolate.

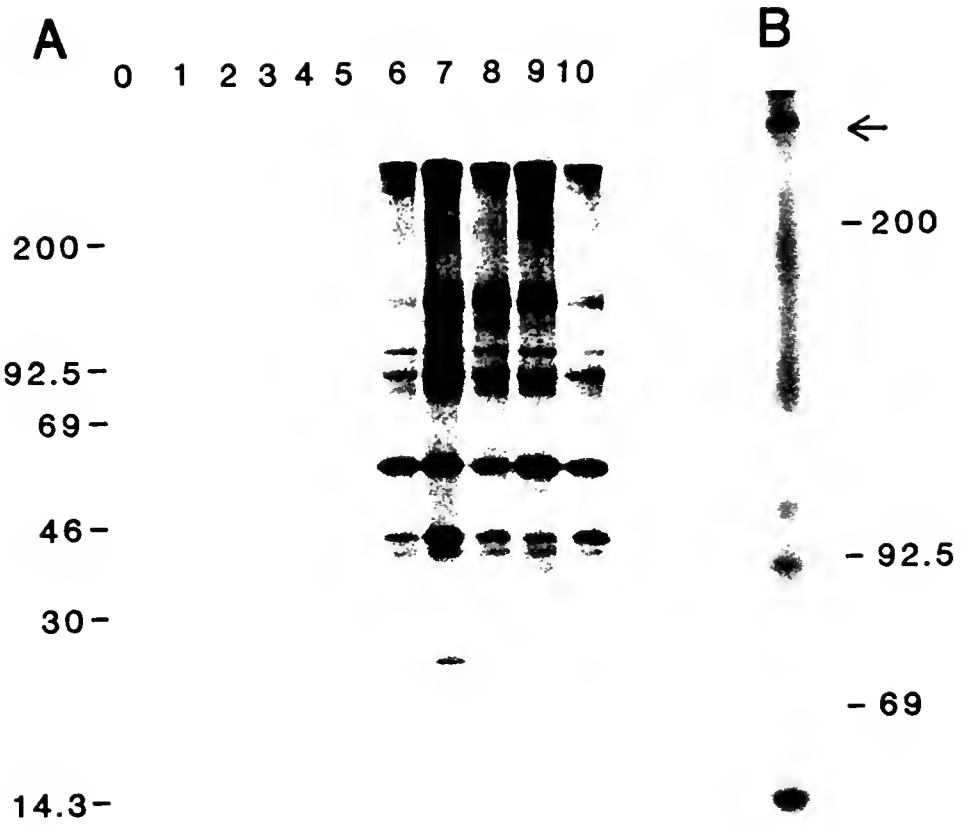


FIGURE 2-2 **Antibodies in immune sera immunoprecipitate eight B. bovis merozoite surface proteins.** **A.** Gradient SDS-PAGE (14 cm.). Radioiodinated merozoite proteins were immunoprecipitated using Protein G-bearing Streptococcus alone (Lane 0), pre-infection sera (Lanes 1-5) or immune serum (Lanes 6-10) from animals B101 (Lane 1,6), B102 (Lane 2,7), B108 (Lane 3,8), B109 (Lane 4,9), and B116 (Lane 5,10). **B.** A 250 kDa radioiodinated protein (arrow) immunoprecipitated by antibodies in immune sera was more clearly resolved in a 25 cm. gradient SDS-polyacrylamide gel.

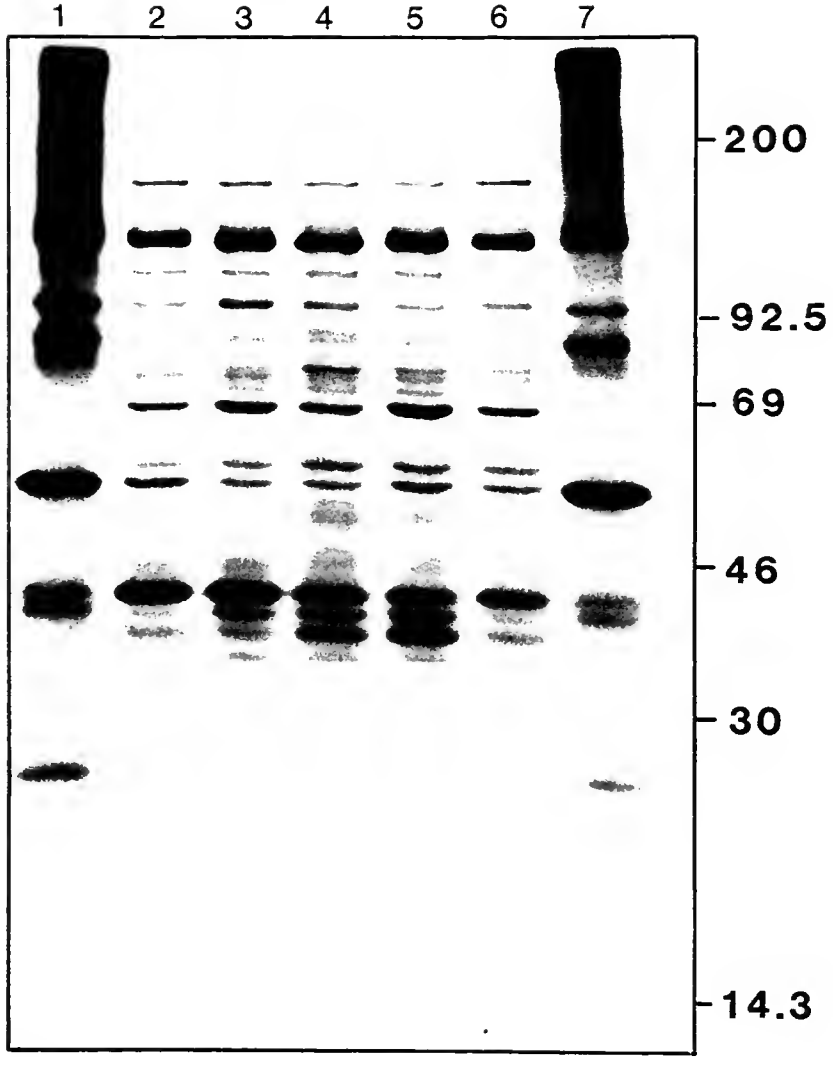


FIGURE 2-3 Surface radiolabeled B. bovis merozoite proteins (Lanes 1,7) comigrate with metabolically labeled antigens (Lanes 2-6). Radioiodinated merozoite surface proteins were immunopreicipitated using immune sera form animals B102 and B116 (Lanes 1 and 7, respectively). ^{35}S-methionine labeled antigens were immunoprecipitated using immune sera from B101 (Lane 2), B102 (Lane 3), B108 (Lane 4), B109 (Lane 5), and B116 (Lane 6).

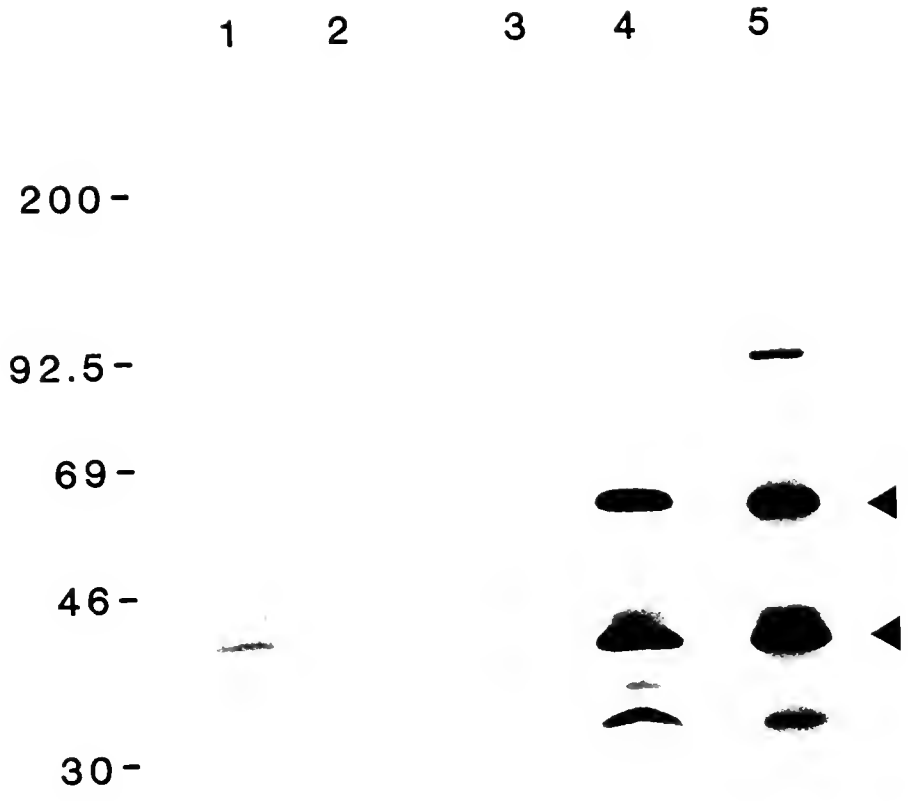


FIGURE 2-4 Post-translational modification of merozoite surface proteins. Antigen was metabolically labeled in erythrocyte cultures with ^{3}H- myristic acid (Lanes 1,2) or ^{3}H-glucosamine (Lanes 3,4). Antibodies in immune serum (animal B116) immunoprecipitated radiolabeled proteins from infected (Lanes 1,4) but not uninfected (Lanes 2,3) cultures. The 42 kDa myristylated B. bovis protein and the 42 and 55 kDa glycosylated proteins comigrated with radioiodinated merozoite surface proteins (arrows) that were immunoprecipitated using immune sera (Lane 5, B109).

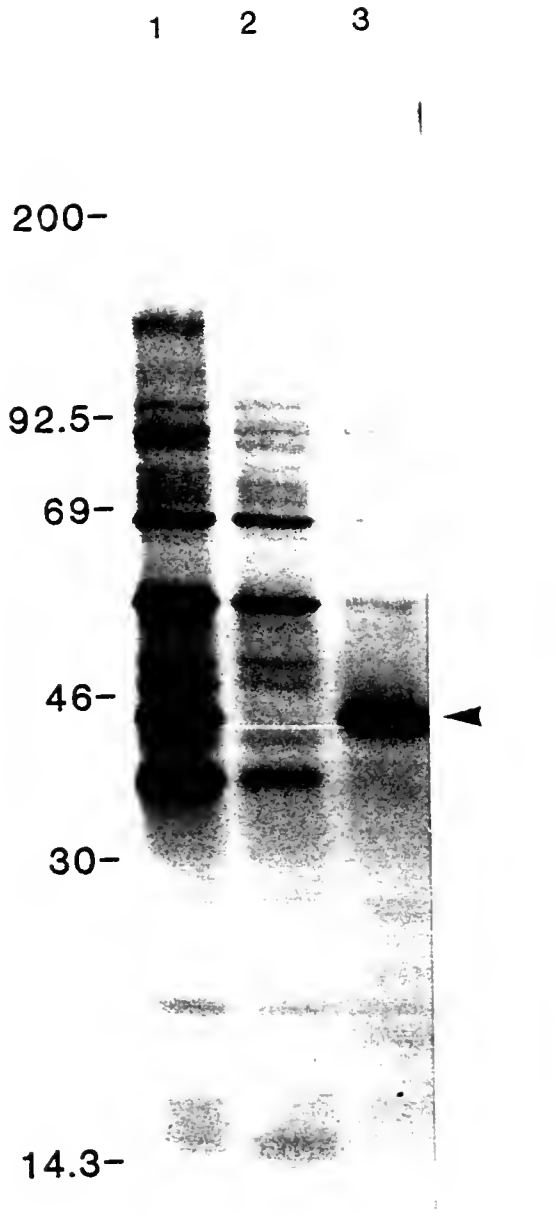


FIGURE 2-5 **The 42 kDa B. bovis merozoite surface protein partitions into the detergent phase of Triton X-114.** Following temperature dependent phase separation, immune serum from animal B101 was used to immunoprecipitate ^{35}S-methionine labeled antigen from the starting 1% Triton X-114 antigen extract (lane 1), the aqueous phase (lane 2) and the detergent phase (lane 3). Arrow indicates the 42 kDa protein.

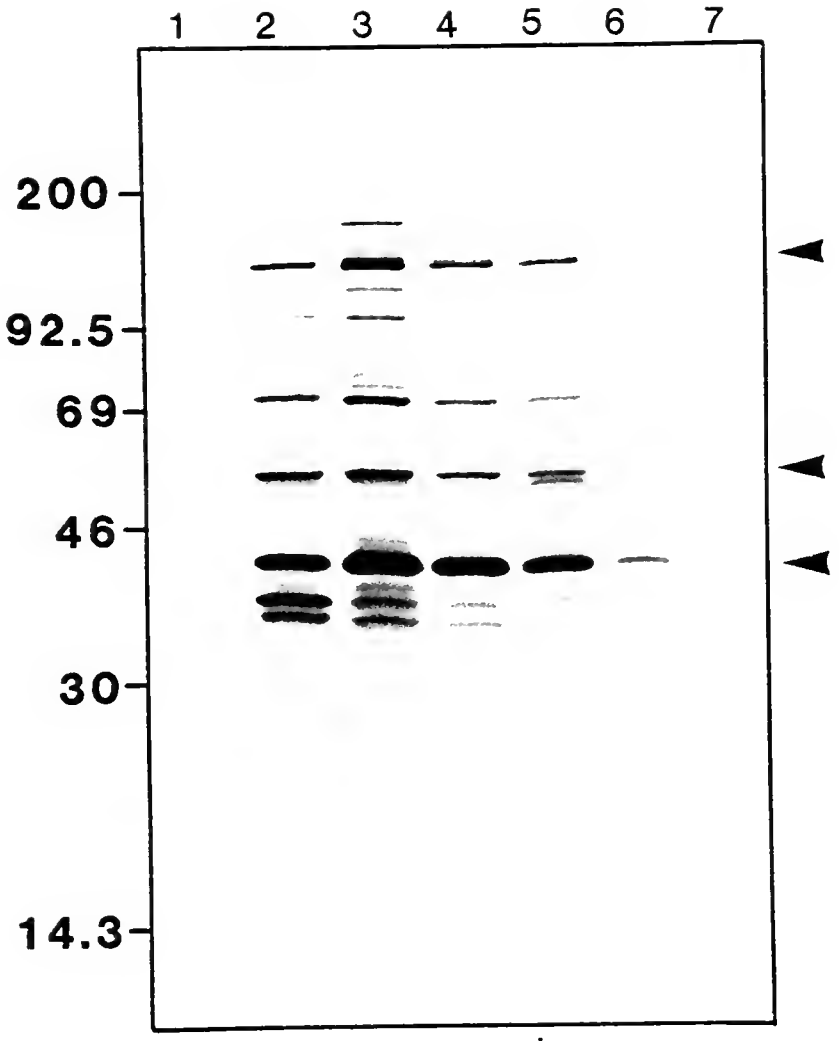


FIGURE 2-6 **Immune cattle produce high titered antibody that is preferentially directed against the 125, 55, and 42 kDa merozoite surface proteins.** ^{35}S-methionine metabolically labeled B. bovis proteins were immunoprecipitated using undiluted preinfection serum from animal B101 (Lane 1) and B101 immune sera that was undiluted (Lane 2) or diluted 1:40, 1:80, 1:160, 1:320, and 1:640 (Lanes 3,4,5,6, and 7, respectively). Arrows indicate the immunodominant 120 kDa, 55 kDa and 42 kDa proteins.

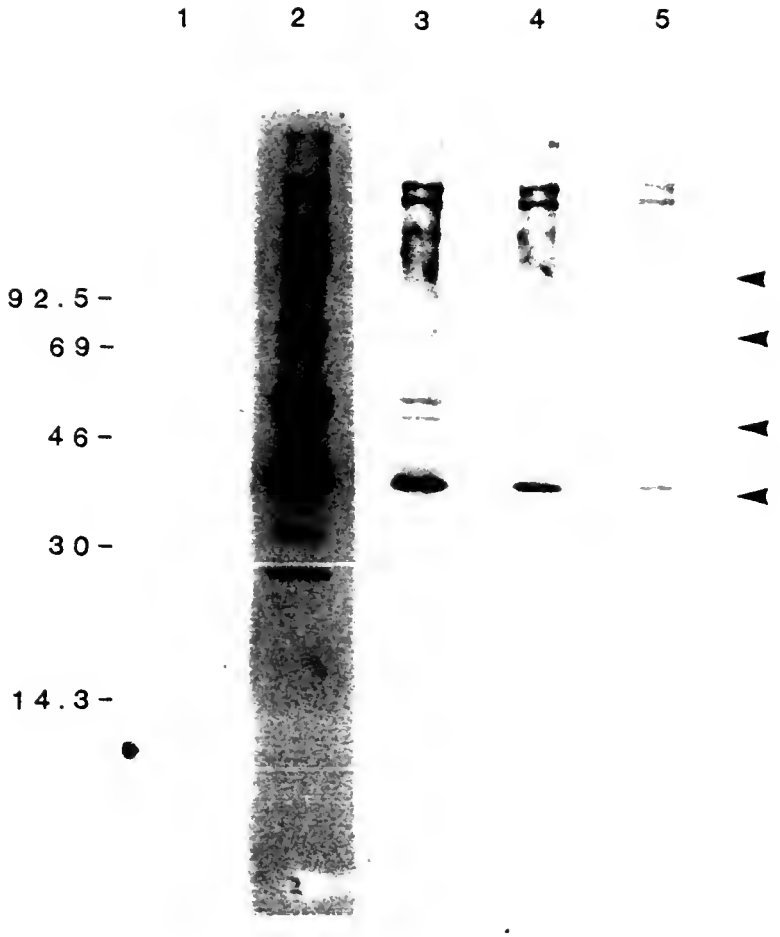


FIGURE 2-7 **Immune cattle produce high titered antibody that preferentially binds the 120, 55, 42, and 85 kDa merozoite surface proteins in immunoblots.** B. bovis blood stage antigen was transblotted and probed using preinfection serum from animal B101 diluted 1:500 (Lane 1) and B101 immune sera diluted 1:500, 1:4000, 1:8000, and 1:16,000 (Lanes 2,3,4, and 5,respectively). Each lane contains 2.5 x 10^7 iRBC. Arrows indicate the 120, 85, 55, and 42 kDa parasite proteins.

CHAPTER 3

MOLECULAR CLONING OF THE GENE ENCODING THE IMMUNODOMINANT *BABESIA BOVIS* MEROZOITE SURFACE PROTEIN Bv42 AND ITS EXPRESSION IN ESCHERICHIA COLI.

Summary

Bv42 is the immunodominant merozoite surface antigen of *Babesia bovis* and a candidate immunogen against bovine babesiosis. A 0.9 kb cDNA fragment encoding Bv42 was cloned into the unique Eco RI site of the bacteriophage vector Lambda ZAP II. The 45 kDa recombinant protein expressed in *Escherichia coli* accounted for greater than 95% of the molecular weight of the native 42 kDa glycoprotein, including the epitope(s) defined by monoclonal antibodies that react with the surface of live merozoites. Antibodies prepared against recombinant Bv42 immunoprecipitated the 42 kDa glycoprotein from surface labeled and metabolically labeled *B. bovis*. Further, antibodies against the recombinant protein bound to and removed the 42 kDa protein recognized by surface reactive monoclonal antibody from a preparation of native, ^{35}S-methionine labeled parasite antigen.

Introduction

Cattle that recover from infection with an attenuated vaccine strain of Babesia bovis are protected against clinical disease induced by arthropod-borne sporozoite challenge [19]. Infection with such an attenuated blood product has serious practical limitations, including variation in infective dose and the potential transmission of other blood borne diseases [74]. However, the observation that an immune response to blood stages protects animals challenged via the arthropod vector suggests strongly that a host immune response to appropriate blood stage antigens would be sufficient to protect cattle against natural Babesia infections. My strategy toward the development of an improved vaccine against Babesia bovis is to identify, isolate, and test antigens exposed on the surface of the merozoite. Merozoite surface proteins are accessible to the host immune system and play a functional role in erythrocyte invasion [75].

Surface reactive antibody that is relevant to protection will likely be present at high levels to ensure antibody-merozoite binding during the parasite's short extracellular phase [65]. Using antibodies from cattle completely protected against clinical babesiosis, I previously identified four immunodominant merozoite surface

antigens having apparent molecular weights of 42, 55, 85, and 125 kDa in SDS-polyacrylamide gels [98]. Bv42 is the surface antigen that elicits the highest antibody titer. This 42 kDa glycoprotein has a surface exposed region defined by monoclonal antibodies [72] and contains epitopes conserved in isolates from Mexico, Honduras, Australia and Argentina [96 and unpublished data]. I report here the molecular cloning and expression of the gene encoding Bv42. Importantly, the recombinant protein expressed in Escherichia coli (E. coli) is a faithful immunologic replica of Bv42 that can be used to induce antibodies against the native glycoprotein.

Materials and Methods

Parasites A cloned Mexico isolate was cultivated in long term cultures by a previously described technique [39]. Parasite antigens were metabolically labeled in vitro with ^{35}S-methionine or ^{3}H-glucosamine as described [98]. Merozoites were isolated from cultures with a high percentage of parasitized erythrocytes [80,79] and surface radioiodinated using the lactoperoxidase technique [83,98]. Merozoite viability was greater than 95% as assessed by retention of 6-carboxyfluoroescein diacetate [81,82].

B. bovis cDNA Expression Library Erythrocytes from asynchronous B. bovis-infected blood cultures were washed three times in Puck's saline G and stored frozen in liquid nitrogen. Cells were thawed in lysis buffer containing 0.2 M NaCl, 0.2 M Tris-HCl pH 7.5, 1.5 mM $MgCl_2$, 2% SDS (w/v), and 200 ug/ml Proteinase K and then incubated in lysis buffer at 46°C for 2 hours [65]. The NaCl concentration of the lysate was adjusted to 0.5 M and poly [A^+] RNA was isolated by batch adsorption with oligo(dT) cellulose [65]. RNA eluted with 0.01 M Tris-HCl pH 7.5 was used to prepare a blood stage cDNA library in Lambda ZAP II (Stratagene, La Jolla, Ca, USA) [70] by a modified Gubler and Hoffman method using Eco RI adapters (Pharmacia LKB, Piscataway, NJ, USA) [67]. The cloned insert in plaque purified lambda phage was subcloned into Bluescript SK(-) phagemid using the in vivo excision capabilities of Lambda ZAP II [70].

Immunoscreening Plaque lifts onto isopropyl thiogalacto-pyranoside soaked nitrocellulose were screened using monospecific rabbit anti-Bv42 antisera (R-914) followed by ^{125}I-Protein A and autoradiography [71,49]. Rabbit R-914 had been immunized with native Bv42 protein immunoaffinity purified using Babb 35A4, a previously described monoclonal

antibody [72]. Positive plaques were tested for reactivity with monoclonal antibodies that recognize a Bv42 surface exposed epitope [72,49] as well as an isotype control monoclonal antibody and normal rabbit sera. Recombinant phagemid excised from positive, plaque purified lambda phage was tested for expression by a similar method using colony lifts from transformed, ampicillin resistant E. coli (XL1-Blue strain) [71].

Restriction Enzyme Digestion Lambda rBv42 phagemid DNA was isolated from bacteria by anion exchange chromatography (Qiagen Inc., Studio City, CA) and restriction enzyme digested by standard methods [99]

Immunoblotting E. coli host strain XL1-Blue containing the lambda rBv42 phagemid, an unrelated recombinant Anaplasma marginale-Bluescript phagemid, or no phagemid was grown in liquid culture to OD_{600}=0.9-1.2. Bacteria washed three times in phosphate-buffered saline (PBS) were lysed in PBS by sonication and several cycles of freeze/thaw. The resulting bacterial lysate was cleared by ultracentrifugation, filtered, and sonicated again before freezing in aliquots at -70°C [83]. Antigen from equivalent numbers of bacteria or

from 2.5 X 10^7 B. bovis infected erythrocytes were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transblotted to nitrocellulose filters [87,98]. Nitrocellulose filters were probed as described for immunoscreening.

Antisera against recombinant Bv42 A whole lysate of E. coli containing lambda rBv42 phagemid was prepared as described above. Two rabbits (R-S19, R-S20) and two 4-5 month old steers were immunized four times with a crude PBS lysate of whole bacteria that had been disrupted by sonication and multiple cycles of freeze/thaw and emulsified in Freund's adjuvant. Two additional rabbits (R-S21 and R-S22) were immunized four times with PBS soluble bacterial antigen that had been cleared by ultracentifugation before emulsification in Freund's adjuvant. Control anti-E. coli antibody was produced by immunizing rabbits with a similar crude PBS lysate of XL1-Blue without Bluescript phagemid (R-126) or XL1-Blue with Bluescript phagemid encoding an unrelated Anaplasma marginale antigen (R-108).

Radioimmunoprecipitation / SDS-PAGE Radiolabeled B. bovis antigen was processed and immunoprecipitated using Protein A-bearing Staphylococcus aureus as previously described

[81,83]. The immunoprecipitates were electrophoresed in 7.5 to 17.5% gradient SDS-polyacrylamide gels and detected by autoradiography [83].

Removal of native Bv42 from an antigen preparation by immunoprecipitation using antibody against rBv42

^{35}S-methionine labeled B. bovis antigen (10^6 protein bound counts per minute/tube) was incubated with 10 ul of rabbit R-S21 antisera or 5 ug of Babb 35A4 monoclonal antibody. Following precipitation of immune complexes with protein A-bearing Staphylococcus aureus, the supernatant antigen from the R-S21 tube was transferred to a new tube and incubated with another 10 ul of R-S21 antisera. Immune complexes were again precipitated using protein A-bearing bacteria. Antigen that remained in the supernatant from this immunoprecipitation was transferred to a third tube and incubated with 5 ug of Babb 35A4. Antigen recognized by monoclonal antibody was precipitated by sequential incubation with rabbit anti-mouse IgG antisera and protein A-bearing Staphylococcus aureus. All immunoprecipitates were eluted from bacteria and examined by SDS-PAGE as described previously [81,83].

Results

Identification of a cDNA clone encoding Bv42 The B. bovis cDNA library, containing 1.2 X 10^6 recombinant plaque forming units, was screened for expression using a monospecific rabbit antiserum (R-914) against Bv42. A positive clone, designated Lambda rBv42, was plaque purified and shown in plaque lifts to express a protein that reacted with Babb 35A4, a monoclonal antibody against native Bv42 that recognizes an exposed epitope on the surface of live merozoites [72]. Positive plaques did not react with normal rabbit serum or isotype control monoclonal antibody.

Lambda rBv42 was subcloned into pBluescript SK(-) phagemid in E. coli XL1-Blue using the in vivo excision properties of Lambda ZAP II [70]. Bacteria containing the recombinant phagemid expressed a protein recognized by R-914 and two surface reactive monoclonal antibodies against native Bv42 (Babb 35A4 and 23.10.36; Fig. 3-1) [6,7]. Positive bacterial colonies were not recognized by normal rabbit serum or control monoclonal antibody, and none of the Bv42 reactive antibodies reacted with host strain bacteria not containing the phagemid.

Size and location of insert The phagemid encoding recombinant Bv42 contained a 0.9 kilobase (kb) DNA insert that was excised as a single fragment with Eco RI. A double restriction digest with Sma I and Kpn I excised a 0.96 kb fragment that migrated just above the Eco RI fragment in agarose gels. Sma I and Kpn I cut pBluescript SK(-) DNA within the polylinker sequence on either side of the Eco RI site and a non-recombinant fragment would be only 0.07 kb. Digestion with other restriction enzymes (Hind III, Xho I, Pvu II, Bam HI, Bgl I, and Pvu I) indicated the presence of a 0.9 to 1.0 kb insert.

Characterization of recombinant protein (rBv42) In immunoblots using R-914 and Babb 35A4, the recombinant *B. bovis* protein expressed in *E. coli* migrated in SDS-polyacrylamide gels at an apparent molecular weight of 45 kDa (Lane 6, Figs. 3-2 A and B), slightly larger than the native glycoprotein (Lane 7, Figs. 3-2 A and B). Neither antibody reacted with proteins produced by *E. coli* (XL1-Blue host strain) without the phagemid or *E. coli* containing an unrelated recombinant Bluescript phagemid. In addition, the recombinant Bv42 protein could not be detected in immunoblots probed with normal rabbit sera or isotype control monoclonal antibody (Figs. 3-2 A and B, Lanes 1-5).

The relative molecular weight of recombinant Bv42 was confirmed by SDS-PAGE after R-914 antisera and Babb 35A4 monoclonal antibody were used to immunoprecipitate from bacterial antigen that had been metabolically labeled in vitro with ^{35}S-methionine.

Antibodies against rBv42 recognize native Bv42 Following immunization with rBv42, all four rabbits and one of two cattle produced antibodies that immunoprecipitated a 42 kDa molecule from native B. bovis antigen (Fig 3-3). The native protein precipitated by antibodies raised against the recombinant protein could be metabolically labeled in infected erythrocyte cultures with ^{35}S-methionine or ^{3}H-glucosamine and was radioiodinated on live merozoites by a surface specific method (Fig 3-4). With each radiolabeling method, the precipitated protein comigrated in SDS-PAGE with a 42 kDa parasite protein immunoprecipitated using the merozoite surface reactive monoclonal antibody Babb 35A4 (Fig. 3-4).

Antibodies from a rabbit immunized with rBv42 were used to deplete a B. bovis antigen preparation of the native 42 kDa surface protein defined by Babb 35A4. A single immunoprecipitation using R-S21 sera precipitated a 42 kDa

protein from ^{35}S-methionine labeled parasite antigen (Fig. 3-5, Lane 2). A second immunoprecipitation from the resulting supernatant using sera from the same rabbit showed that none of the 42 kDa protein recognized by antibodies in R-S21 sera remained (Fig. 3-5, Lane 3). A radiolabeled protein was also not detected when the depleted supernatant antigen was subsequently precipitated using Babb 35A4 (Fig. 3-5, Lane 4). In contrast, the same antigen preparation not previously depleted with antibodies against rBv42 contained a 42 kDa protein that could be immunoprecipitated using Babb 35A4 (Fig. 3-5, Lane 5).

Discussion

Bv42 is the immunodominant B. bovis merozoite surface antigen, preferentially recognized by antibodies from cattle protected against babesiosis [98]. This 42 kDa integral membrane glycoprotein can be surface radiolabeled and was originally defined by monoclonal antibody Babb 35A4 that bound to an exposed epitope on live merozoites [72]. In addition, Bv42 has previously been shown to be species specific and to contain epitopes conserved in at least four geographic isolates [96 and unpublished data]. These features make Bv42 a strong candidate for inclusion into a subunit vaccine against B. bovis.

The marked immunodominance of Bv42 and conservation of epitopes in multiple isolates suggests also that this protein will be a valuable diagnostic reagent. Affinity purified rBv42 or a synthetic peptide corresponding to the immunodominant region of the native protein would provide a B. bovis-specific target antigen for use in an antibody based diagnostic test [96].

B. bovis cDNA cloned into Lambda ZAP II and subsequently subcloned into Bluescript SK(-) phagemid encoded a recombinant parasite protein that is a faithful immunologic replica of the native molecule. The recombinant protein bears the Bv42 surface epitope defined by the monoclonal antibody Babb 35A4, and antisera generated against the recombinant protein immunoprecipitated the 42 kDa native B. bovis protein. It was important to prove definitively that the 42 kDa protein precipitated by antibodies raised against the recombinant was the same 42 kDa surface protein defined by Babb 35A4. Both antigens could be metabolically labeled in vitro with ^{3}H-glucosamine and surface labeled on live merozoites with ^{125}I, techniques by which a limited number of parasite proteins are radiolabeled [98]. More directly, however, antibodies against rBv42 bound to and removed the glycoprotein

recognized by Babb 35A4 from a preparation of native antigen.

Based on its molecular weight, the recombinant protein is predicted to include greater than 95% of native Bv42. In SDS-polyacrylamide gels, recombinant Bv42 migrated just above the native protein at a relative molecular weight of 45 kDa. Expression of a gene cloned into the unique Eco RI site of Lambda ZAP II should produce a fusion protein containing 3.9 kDa of vector encoded polypeptide (Stratagene). Since Bv42 would not be glycosylated in a prokaryotic vector, rBv42 would likely migrate at a lower relative molecular weight than the native glycoprotein. Therefore, the 45 kDa rBv42 described here is predicted to include at least 41 kDa of the 42 kDa native molecule.

Restriction enzyme mapping of isolated phagemid DNA confirmed that the cDNA insert was cloned into the unique Eco RI site of the vector. A 0.9 kb fragment was excised using Eco RI and restriction enzymes that cut within the multiple cloning site on either side of the Eco RI site. An insert of this size should encode a protein of only about 30 kDa, but many parasite antigens migrate anomalously in SDS-PAGE at higher molecular weights than predicted due to highly repetitive primary sequences [100]. These repetitive regions also tend to be immunodominant and may explain the

marked antibody response to Bv42 in cattle infected with B. bovis [101]. The apparent discrepancy between the size of the insert and the relative molecular weight of the recombinant protein in SDS-PAGE will be resolved by sequencing this gene.

The cloning of Bv42 will now enable testing of the hypothesis that a monospecific immune response against this merozoite surface antigen will protect cattle against babesiosis. The recombinant protein expressed in E. coli contains at least one epitope exposed on live merozoites and has already been shown to induce antibody that recognizes native Bv42. That recombinant protein is being isolated by immunoaffinity chromatography and will be used to immunize cattle prior to challenge with B. bovis. In addition, the gene for Bv42 will be subcloned into vaccinia virus for testing as an immunogen. Expression in vaccinia virus should result in appropriate glycosylation and folding of rBv42, thereby preserving potentially important epitopes contributed by carbohydrate moieties or tertiary structure [73].

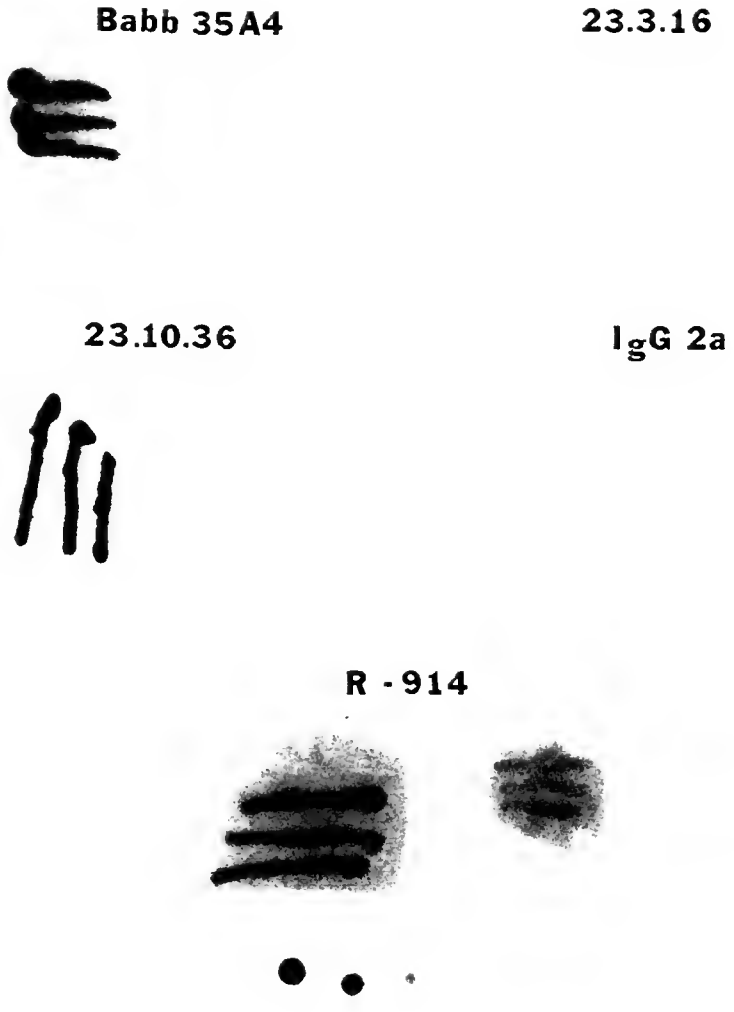
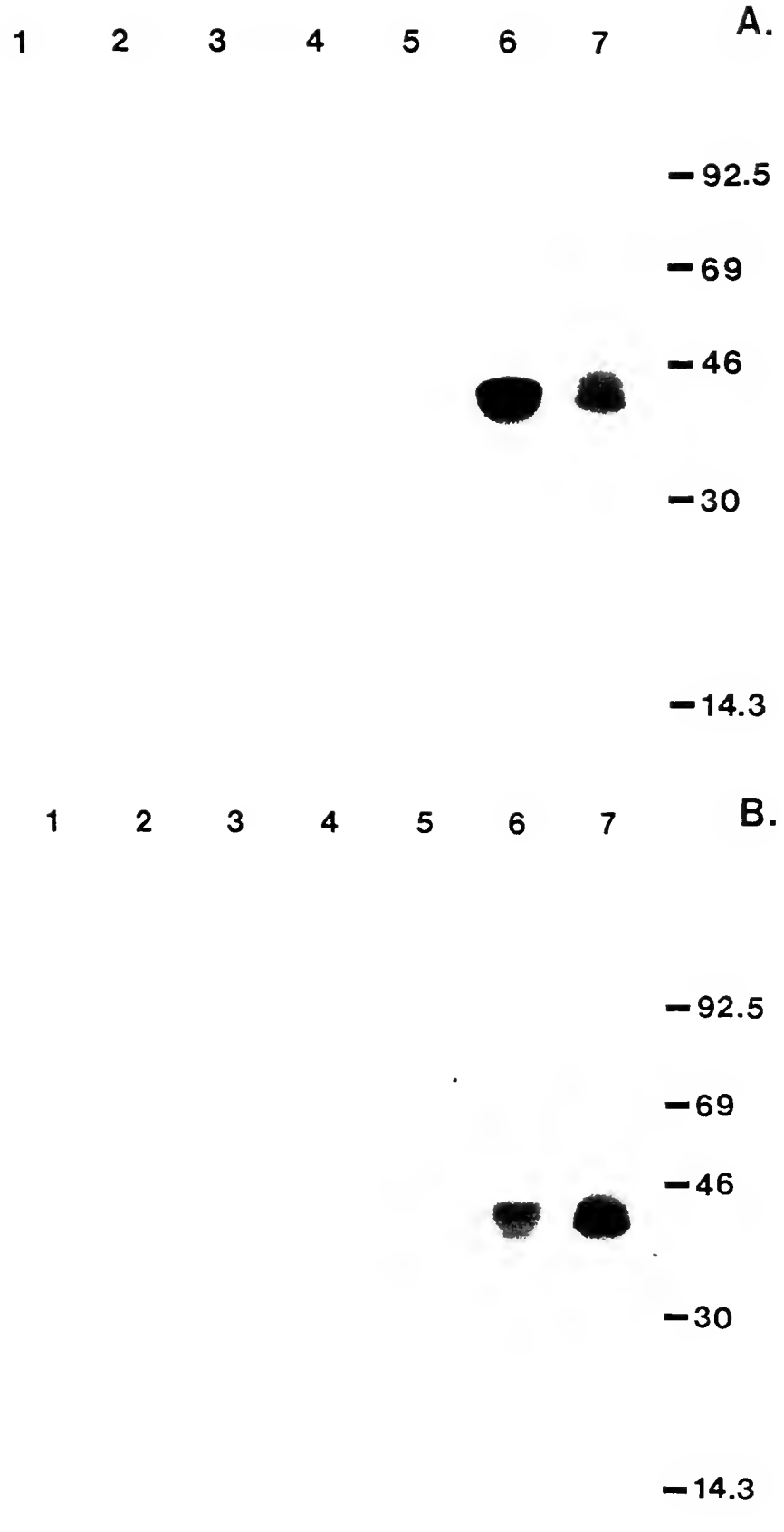


FIGURE 3-1 **E. coli containing the recombinant phagemid express a protein recognized by two surface reactive monoclonal antibodies against native Bv42 and by rabbit anti-serum (R-914) that is monospecific for Bv42.** Colony lifts from XL-1 Blue host strain bacteria (right) or XL-1 Blue containing the recombinant phagemid (left) were probed with three monoclonal antibodies that react with the surface of live merozoites (Babb 35A4, 23.10.36, and 23.3.16), an IgG_{2a} monoclonal antibody isotype control, or R-914 anti-serum. As a positive control, R-914 was also used to detected B. bovis-infected erythrocytes that were spotted onto nitrocellulose membranes at 10^6/1 ul, 10^5/1 ul, 10^4/1 ul, and 10^3/1ul (left to right).

FIGURE 3-2 **E. coli containing the recombinant Bv42 phagemid express a 45 kDa protein bearing the surface exposed epitope defined by monoclonal antibody Babb 35A4.** Antigen was collected from the XL1-Blue E. coli host strain (Lanes 1 and 4), XL1-Blue containing a recombinant Bluescript SK(-) phagemid encoding an Anaplasma marginale gene (Lanes 2 and 5), and XL1-Blue containing the rBv42-Bluescript phagemid (Lanes 3 and 6). Bacterial antigen (Lanes 1-6) or B. bovis iRBC antigen (Lane 7) were transblotted and probed with normal rabbit sera (Fig. A, Lanes 1-3), monospecific rabbit anti-Bv42 sera R-914 (Fig. A, Lanes 4-7), an IgG_{2a} isotype-control monoclonal antibody control (Fig. B, Lanes 1-3), or Babb 35A4 (Fig. B, Lanes 4-7).


1 2 3 4 5 6 7
A.
92.5
69
46
30
14.3
1 2 3 4 5 6 7
B.
92.5
69
46
30
14.3

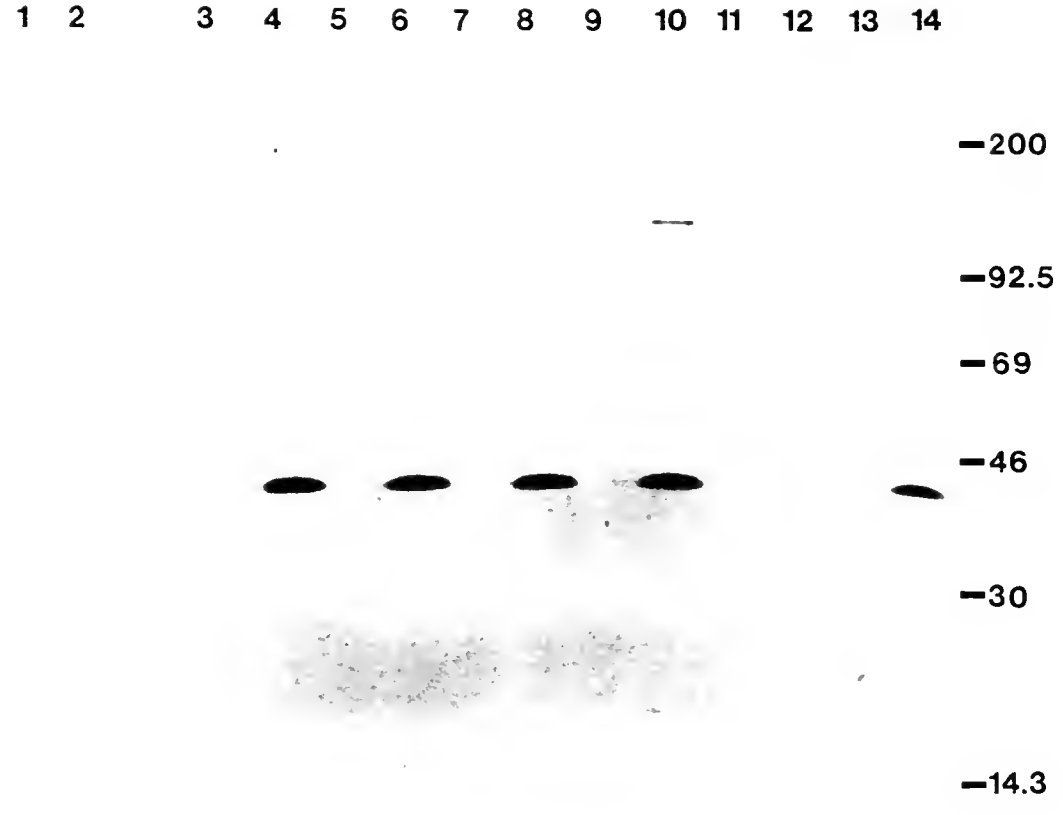


FIGURE 3-3 **Antibodies against the recombinant Bv42 protein immunoprecipitate a 42 kDa native protein from ^{35}S-methionine labeled B. bovis antigen.** Metabolically labeled protein was immunoprecipitated using pre-immunization sera (odd numbered lanes) or post-immunization sera (even numbered lanes) from rabbits R-S19 (Lanes 3,4), R-S20 (Lanes 5,6), R-S21 (Lanes 7,8), and R-S22 (Lanes 9,10) and cattle B-770 (Lanes 11,12) and B-783 (Lanes 13,14). Parasite antigen was also precipitated using sera from control rabbits immunized with lysates of E. coli host strain XL1-Blue (R-126, Lane 2) or XL1-Blue containing an A. marginale recombinant phagemid (R-108, Lane 1).

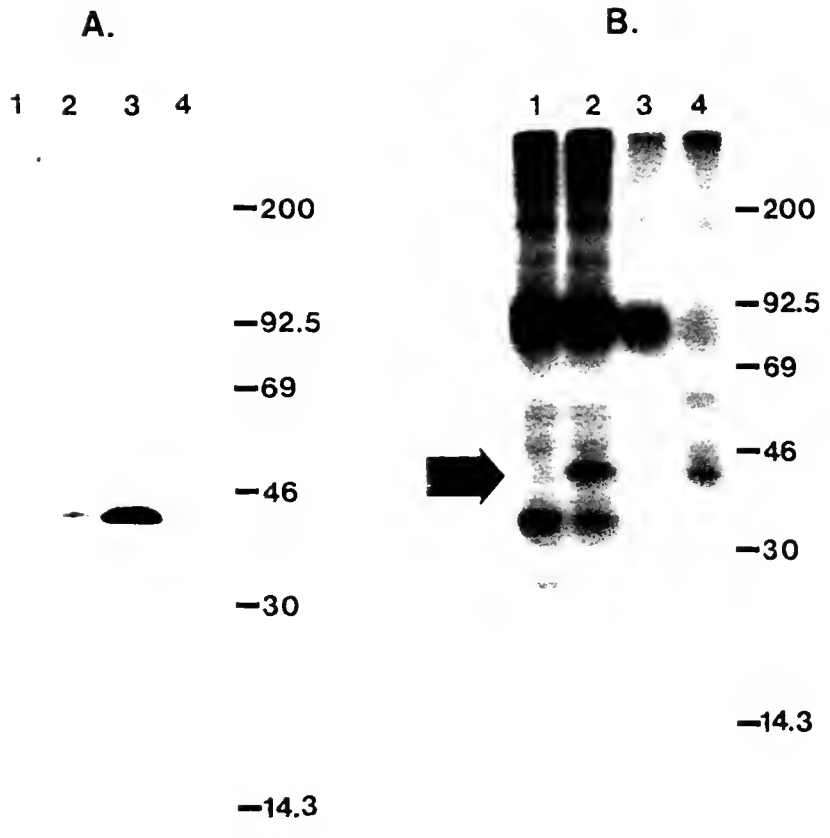


FIGURE 3-4 **Bovine antibodies against the recombinant Bv42 protein immunoprecipitate a 42 kDa native protein that is metabolically labeled with ^{3}H-glucosamine, surface labeled on B. bovis merozoites, and co-migrates with the surface protein defined by Babb 35A4.** **A.** Parasite antigen was labeled *in vitro* with ^{3}H-glucosamine and immunoprecipitated using pre-immunization sera from steer B-783 (Lane 1), post-immunization sera from B-783 (Lane 2), Babb 35A4 (Lane 3), or an IgG_{2a} control monoclonal antibody (Lane 4). **B.** Radioiodinated merozoite surface proteins were immunoprecipitated using pre-immunization sera from B-783 (Lane 1), post-immunization sera from B-783 (Lane 2), an IgG_{2a} control monoclonal antibody (Lane 3), and Babb 35A4 (Lane 4). Arrow indicates the ^{125}I-labeled 42 kDa native B. bovis protein. With both antigen preparations, identical results were obtained using rabbit anti-rBv42 sera (data not shown). Immunoprecipitations using sera from control rabbits immunized with E. coli (R-126) or E. coli containing a control phagemid (R-108) revealed no specific bands on SDS-PAGE.

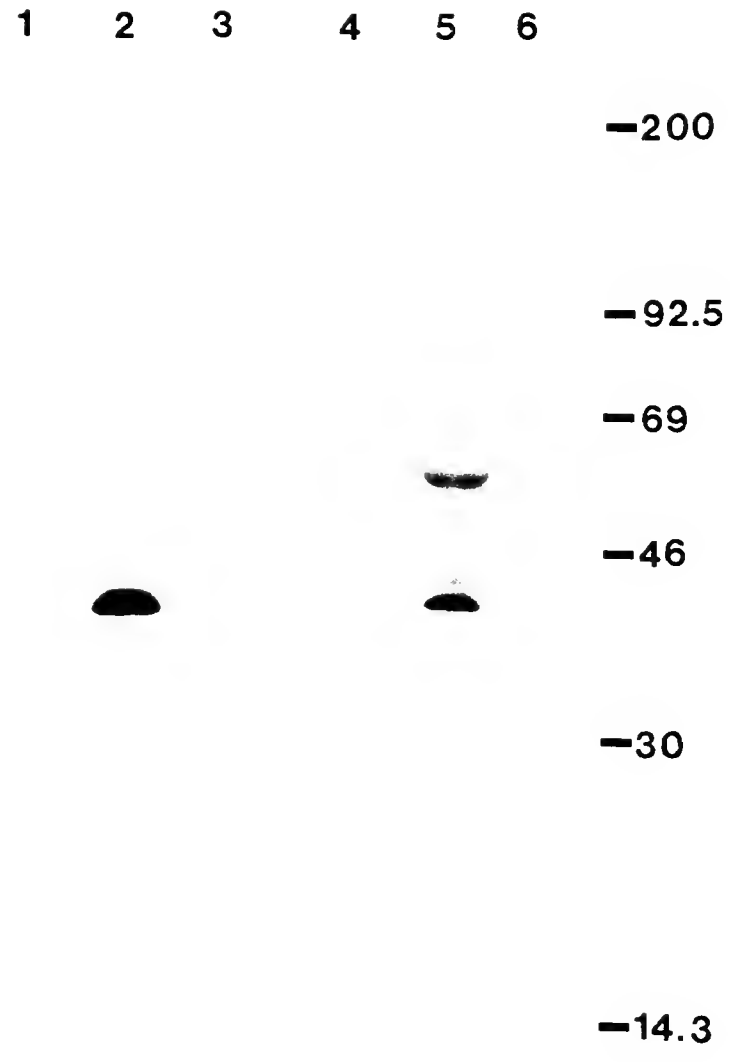


FIGURE 3-5 **Antibodies against the recombinant Bv42 protein bind to and remove the glycoprotein recognized by Babb 35A4 from a preparation of native antigen.** ^{35}S-methionine labeled antigens of B. bovis contain a 42 kDa protein that is immunoprecipitated using R-S21 rabbit anti-rBv42 antibodies (Lane 2) or Babb 35A4 (Lane 5), but not using R-S21 pre-immunization sera (Lane 1) or an IgG_{2a} control monoclonal antibody (Lane 6). One immunoprecipitation using R-S21 antibodies completely removes the 42 kDa protein from an antigen preparation so that it can no longer be immunoprecipitated using additional R-S21 antibodies (Lane 3) or Babb 35A4 monoclonal antibody (Lane 4).

CHAPTER 4

SUMMARY \ CONCLUSIONS

Merozoite surface antigens are potential targets of the protective immune response to Babesia bovis and, therefore, candidates for an improved vaccine. Antibody directed against surface exposed regions of merozoite antigens is likely to inhibit recognition and invasion of erythrocytes. Alternatively, antibody against the merozoite surface could opsonize the parasite for phagocytosis or, in conjunction with complement, mediate merozoite lysis.

Using antibodies from cattle which are protected against babesiosis, I have identified eight surface-radiolabeled merozoite proteins from a cloned, pathogenic B. bovis isolate. Among these eight surface proteins, the 55 and 42 kilodalton molecules were glycoproteins that could be biosynthetically labeled with ^{3}H-glucosamine. The 42 kDa glycoprotein, designated Bv42, could also be labeled with ^{3}H-myristic acid and partitioned into the detergent phase in Triton X-114 extracts, indicating that it was an integral membrane protein and suggesting that it was anchored by a glycosylphosphatidylinositol moiety. This information may

be important for the presentation of Bv42 as an immunogen. Native or recombinant protein may need to be presented in the context of a membrane to generate antibody that reacts with the antigen as it is expressed on the surface of the merozoite.

Antibody mediated protection against B. bovis merozoites most likely requires a high level of circulating antibody to ensure antibody-merozoite binding during the parasite's transient extracellular phase. Antibodies in diluted sera from protected cattle selectively recognize the 120, 85, 55, and 42 kDa surface proteins. Only the 42 kDa integral membrane glycoprotein Bv42 is reactive with serum antibodies diluted $\geq$ 1:16,000. I hypothesized that the immunodominant proteins, especially Bv42, were important to the induction of the protective immune response and set out to test that hypothesis by first cloning the genes encoding one or more of these candidate immunogens. Cloning and expression will provide parasite antigen in sufficient quantity and purity to test in immunization trials.

A 0.9 kb cDNA fragment encoding the 42 kDa immunodominant merozoite surface antigen Bv42 was cloned into the unique Eco RI site of the bacteriophage vector Lambda ZAP II. Expression of recombinant DNA in the multiple cloning site of the lac Z gene of Lambda ZAP II

should produce a fusion protein containing only 3.9 kDa of vector encoded polypeptide. The 45 kDa recombinant B. bovis protein expressed in Escherichia coli accounted for greater than 95% of the molecular weight of the native 42 kDa glycoprotein and, importantly, included the epitope(s) defined by monoclonal antibodies that react with the surface of live merozoites.

Antibodies were prepared against the recombinant Babesia protein to prove definitively that the cloned gene encoded Bv42 and to show that antibodies directed against the recombinant protein recognize the native parasite protein. This second point, that the recombinant protein functions as an immunologic replica of the native protein, is critical if recombinant Bv42 (rBv42) is to be tested as an immunogen for babesiosis. Antibodies from animals immunized with rBv42 immunoprecipitated the 42 kDa glycoprotein from surface labeled and metabolically labeled B. bovis. Further, antibodies against the recombinant protein bound to and removed the 42 kDa protein recognized by surface reactive monoclonal antibody Babb 35A4 from a preparation of native, methionine labeled parasite antigen.

A successful immunization trial using an isolated B. bovis antigen would focus the efforts to develope an improved vaccine. In addition, however, the work to isolate

the genes for B. bovis antigens and develop monospecific reagents will help answer important questions about the basic biology of Babesia spp. Those questions will include the biologic function of merozoite surface antigens, the extent of antigenic diversity among different geographic isolates, the role, if any, of antigenic variation within a single clone of parasites, and the genetic mechanisms involved in the expression and modification of these important parasite molecules.

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BIOGRAPHICAL SKETCH

Stephen Alan Hines was born to Della Maxene Hanna Hines and Earl Thomas Hines on March 5, 1955, in Bowling Green, Kentucky. He received a Bachelor of Arts in May, 1977, from Miami University in Oxford, Ohio. On June 12, 1981, Steve graduated as a Doctor of Veterinary Medicine from Ohio State University, College of Veterinary Medicine. The following day he married Dr. Melissa Gail Trogdon, also a veterinarian. At that time Steve inherited Riley, a once-in-a-lifetime dog.

After a year of dairy and small animal practice, Steve moved with Melissa to Gainesville, Florida, to start an anatomic pathology residency at the University of Florida's Veterinary Medical Teaching Hospital. With his residency completed in July, 1984, Steve entered graduate school in the College of Medicine, University of Florida. In September, 1985, Steve passed his veterinary pathology specialty boards. Six months later, he changed major advisors to work on the immunology and molecular biology of veterinary hemoparasites, in particular Babesia bovis, with Guy Palmer. When Guy left Florida in May, 1988, to be

closer to mountains, Steve moved to Terry McElwain's laboratory to continue his dissertation work on Babesia. In June, 1989, Terry also left Florida to return to the same place Guy went. Soon Steve and Melissa will be following Terry and Guy to the mountains - to faculty positions at Washington State University, that is. It's been great being a gator, now let's try being a cougar!

After we're gone, try to preserve the natural beauty of Florida. It's worth fighting for.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Terry F. McElwain, Chair
Assistant Professor of Veterinary Medicine

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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